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Journal of Applied Physiology 99:1769-1775, 2005. First published Jul 7, 2005;
doi:10.1152/japplphysiol.00010.2005

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Submitted 4 January 2005; accepted in final form 7 July 2005

Rassier, Dilson E., and Walter Herzog. Relationship between force and stiffness in muscle fibers after stretch. *J Appl Physiol* 99: 1769–1775, 2005. First published July 7, 2005; doi:10.1152/japplphysiol.00010.2005.—The purpose of this study was to evaluate the relationship between force and stiffness after stretch of activated fibers, while simultaneously changing contractility by interfering with the cross-bridge kinetics and muscle activation. Single fibers dissected from lumbrical muscles of frogs were placed at a length 20% longer than the plateau of the force-length relationship, activated, and stretched by 5 and 10% of fiber length (speed: 40% fiber length/s). Experiments were conducted with maximal and submaximal stimulation in Ringer solution and with the addition of 2 and 5 mM of the myosin inhibitor 2,3-butanedione monoxime (BDM) to the solution. The steady-state force after stretch of an activated fiber was higher than the isometric force produced at the corresponding length in all conditions investigated. Lowering the frequency of stimulation decreased the force and stiffness during isometric contractions, but it did not change force enhancement and stiffness enhancement after stretch. Administration of BDM decreased the force and stiffness during isometric contractions, but it increased the force enhancement and stiffness enhancement after stretch. The relationship between force enhancement and stiffness suggests that the increase in force after stretch may be caused by an increase in the proportion of cross bridges attached to actin. Because BDM places cross bridges in a weakly bound, pre-power-stroke state, our results further suggest that force enhancement is partially associated with a recruitment of weakly bound cross bridges into a strongly bound state.

cross bridge; muscle contraction; force enhancement; 2,3-butanedione monoxime

WHEN SKELETAL MUSCLE IS STRETCHED while activated, the steady-state isometric force attained after stretch is higher than that produced during purely isometric contractions at the corresponding length (1, 8, 14, 22, 25, 28). The mechanism behind this phenomenon, referred to as residual force enhancement, remains unknown. It has been suggested that force enhancement is associated with cross-bridge kinetics (26), specifically that the proportion of cross bridges attached to actin after stretch is increased compared with that attained during isometric contractions (14, 20). Assuming that everything else remains the same, an increased proportion of attached cross bridges would be associated with an increased stiffness (11).

Sugi and Tsuchiya (31) showed that stiffness of single fibers increased during stretch of activated fibers, and then decreased just after stretch, reaching the same level as that observed during isometric contractions at the corresponding length. Using a similar preparation, Julian and Morgan (18) showed that stiffness increased during stretch and remained virtually constant after stretch, not decreasing to the level of the isometric contractions just before stretch. Although these results are different, both authors used them to dismiss cross-bridge explanations for the force enhancement. However, in a study using whole cat soleus, it was found that stiffness was increased in the force-enhanced state compared with isometric contractions at the corresponding length (14). Therefore, stiffness results between studies and across different preparations are inconclusive.

The purpose of this study was to evaluate the relationship between force and stiffness after stretch of activated single fibers, while simultaneously changing contractility by changing muscle activation and cross-bridge kinetics. Activation was altered by changing the frequency of stimulation, and cross-bridge kinetics was altered by adding the myosin inhibitor 2,3-butanedione monoxime (BDM) to the Ringer solution. Decreasing frequency of stimulation decreases force and stiffness simultaneously, whereas BDM reduces force while leaving stiffness nearly unaffected by biasing the ratio of strongly bound vs. weakly bound cross bridges toward the weakly bound state (13, 29).

METHODS

Muscle fiber preparation. Single muscle fibers with an approximate length of 2 mm were carefully dissected from lumbrical muscles of the foot of the frog *Rana pipiens*. Treatment of these animals and all experimental procedures were approved by the University of Calgary committee for the ethical use of animals in research.

The tendons of the isolated fibers were gripped with T-shaped, aluminum foil clips, as close as possible to the tendons to minimize compliance of the system. The two clips were hooked between a servomotor length controller (Aurora Scientific) and a force transducer (Sensomotor) inside an experimental chamber, containing temperature-controlled (~9°C) Ringer solution (in mmol: 115 NaCl, 3 KCl, 3 $CaCl_2$, 2 NaH_2PO_4, and 20 $NaHCO_3$, pH = 7.5). The fiber was then suspended inside the experimental chamber between two platinum wire electrodes, connected to a stimulator (Grass S88, Grass Instruments). Fibers were activated through electrical stimulation using square-wave pulses (0.4-ms duration) at an amplitude of 25% above the voltage that produced maximal tetanic force production (range: 25–50 V). The frequency of stimulation was set individually for each fiber to produce a fused tetanic contraction with the smallest possible frequency to avoid fatigue and/or damage (range in these experiments: 25–35 Hz).

Fiber length and force measurements. After defining the optimal voltage and frequency of stimulation for the experiments, fibers were paced for ~60 min with twitch contractions (90-s intervals). During this period, fibers were inspected visually for any apparent damage, and they were evaluated for a decrease in force. After the pacing, two to three tetanic contractions (1 s) were performed to check for any decrease in force. If damage was found or force had decreased, the fibers were discarded and the experiments were stopped. Reference

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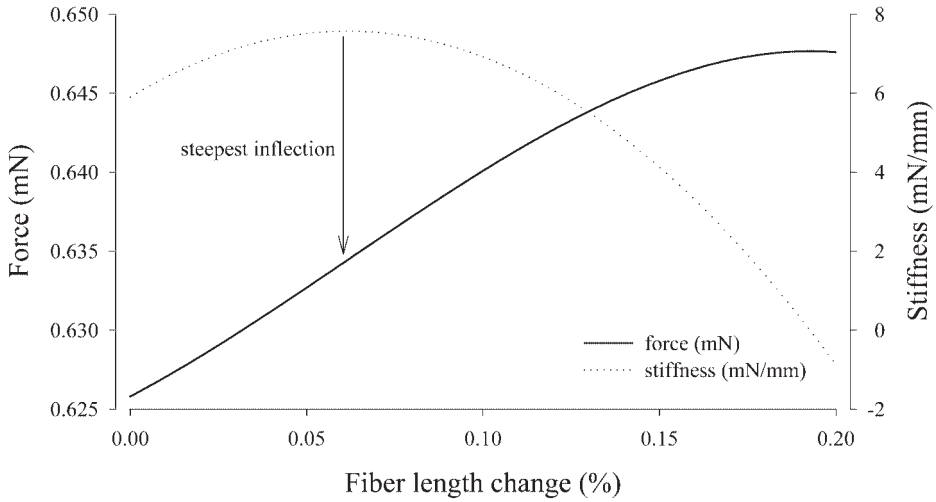

Fig. 1. Stiffness measurement during a typical experiment. Force trace during length change imposed to the fiber during an isometric contraction is shown. Force-length curve was fitted with a third-order exponential equation, and the derivative of the fitted curve was taken to define maximal stiffness, corresponding to the steepest point of the force-extension curve. In this example, the force curve was fitted as follows: $y = -367{,}165 \cdot x^3 + 1{,}356.3 \cdot x^2 + 5.8{,}982 \cdot x + 0.6{,}258$; $r^2 = 0.99$.

contractions (1 s) were repeated throughout the experiments, and testing was stopped at any time if the reference force was decreased.

An active force-length relationship was obtained from isometric tetanic contractions (2-s duration, 5-min intervals). The plateau of the force-length relationship, referred to as L_o hereafter, and the descending limb of the force-length relationship were identified. Fibers were then placed at an initial length (L_i) of ~20% above L_o, and they were activated to produce maximal isometric force. At 1 s after the onset of activation, fibers were stretched (5 and 10% of fiber length, at a speed of 40% fiber length/s) and held isometric at the final length (L_f). The total contraction time was always 4 s. At all times, isometric reference contractions were performed at L_o, L_i, and L_f before and after the stretch contractions. In all (isometric and stretch) contractions, a stretch of 0.2% fiber length at 100% fiber length/s was imposed just before deactivation to measure stiffness of the fibers.

To test the relationship between force enhancement and stiffness for different cross-bridge kinetics, all procedures described above were repeated, in the same fibers, after adding 2 mM and 5 mM BDM to the Ringer solution, and using contractions performed at a frequency of 50 and 25% of the frequency necessary to produce a fused tetanic contraction.

Data analysis. Force enhancement was defined as the increase in steady-state force after active stretch compared with the purely isometric force at the corresponding length. Tangential stiffness was measured by taking the derivatives of the force increase during the imposed length change. Therefore, the measurement reflects the instantaneous stiffness (Fig. 1). Force and stiffness after the active stretch tests and the isometric reference contractions for the different conditions were compared using nonparametric statistics. A significance level of $P < 0.05$ was used for all analyses.

RESULTS

Force and stiffness of the single fibers investigated in this study were significantly affected by BDM, length, and frequency of stimulation (Tables 1–3). Increasing concentrations of BDM and decreasing frequencies of stimulation decreased the active force produced during isometric contractions at all lengths investigated (Table 1). Increasing lengths decreased the active force produced during isometric contractions in Ringer solution, after adding 2 mM BDM, or when the stimulation frequency was lowered to 50% of its tetanic level. After adding 5 mM BDM, or lowering the stimulation frequency to 25% from its tetanic level, the active force during isometric contractions was not decreased significantly with increasing fiber lengths (Table 1).

Stiffness recorded at L_o was (mean ± SD) 7.58 ± 1.41, 7.44 ± 1.48, 5.45 ± 0.44, and 6.62 ± 1.23 mN/mm for isometric contractions performed in Ringer solution, with 2 mM and 5 mM BDM, and with 50% of the tetanic frequency of stimulation, respectively. There was a distinct difference between the effects of BDM on force and stiffness, because BDM decreased force significantly more than it decreased stiffness (Fig. 2).

The steady-state isometric force after stretch of fibers activated in Ringer solution was higher than the force produced during the isometric reference contractions at the corresponding lengths (Fig. 3). This force enhancement was greater after 10% compared with 5% stretches (Fig. 3, Table 2). Fiber stiffness in the force-enhanced state was higher than stiffness obtained for the isometric reference contractions (Table 3).

Adding BDM decreased isometric force, but it increased the relative force enhancement. This increase was higher after the 10% compared with the 5% stretch (Fig. 4, Table 2). Caution should be exercised when interpreting these results, because force did not reach a complete steady state for the tests involving BDM, because force relaxation after stretch was very slow. After BDM administration, fiber stiffness decreased, but stiffness was always greater in the force-enhanced state compared with the isometric reference state (cf. Fig. 4; Table 3).

When fibers were stretched during submaximal stimulation (50 and 25% of the tetanic stimulation frequency), force

Table 1. *Active force produced during isometric contractions in Ringer solution, with 2 mM and 5 mM BDM, and with 50% and 25% of the tetanic frequency of stimulation + 25% and L_o + 30%*

	L_o	L_o + 20%	L_o + 25%	L_o + 30%
Control	0.58±0.11	0.54±0.10	0.52±0.09	0.49±0.10
2 mM BDM	0.46±0.14	0.42±0.07	0.41±0.07	0.39±0.07
5 mM BDM	0.26±0.07	0.27±0.07	0.26±0.07	0.26±0.08
50% Fr	0.51±0.09	0.48±0.09	0.47±0.08	0.43±0.08
25% Fr	0.36±0.07	0.36±0.07	0.34±0.06	0.35±0.06

Values are means ± SD. Contractions were performed isometrically (without changing the length during the contractions) at optimal length (L_o), L_o + 20%, L_o + 25% and L_o + 30% given in mN. BDM, 2,3-butanedione monoxime; 50% Fr, 50% of tetanic frequency of stimulation; 25% Fr; 25% of tetanic frequency of stimulation.

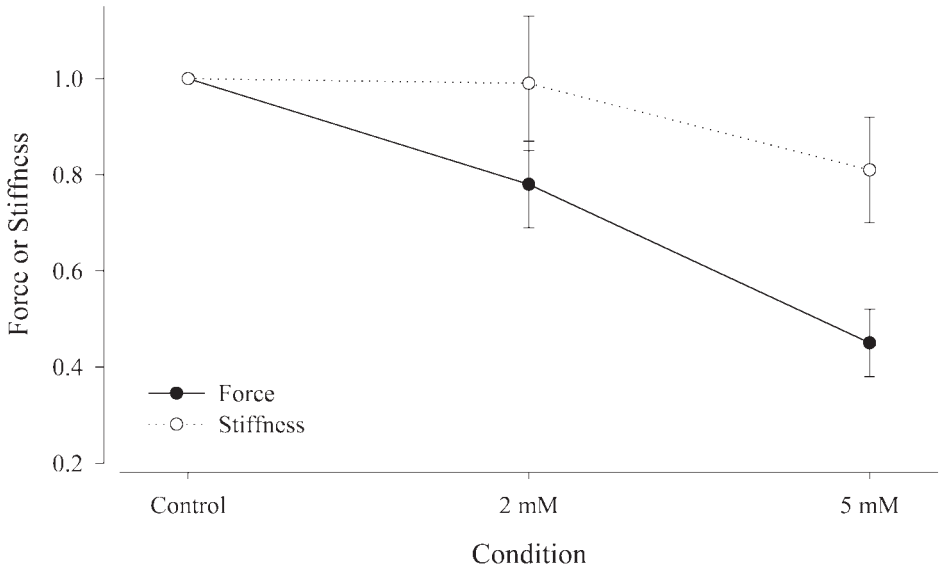

Fig. 2. Effects of 2,3-butanedione monoxime on isometric force and stiffness, during contractions performed at optimal length (L_o). Force and stiffness were normalized to the maximal values obtained during individual contractions. Note that 2,3-butanedione monoxime causes a greater decrease in force than stiffness. Values are means ± SD.

enhancement was similar to that observed for maximally stimulated fibers (Figs. 5 and 6, Table 2). At 50% stimulation, fiber stiffness was greater in the force-enhanced compared with the isometric reference state (Fig. 5, Table 3). No reliable stiffness measurements could be obtained for stimulations at 25% of the tetanic level.

DISCUSSION

The main findings of this study were that *1*) force enhancement was accompanied by an increased stiffness compared with the isometric reference stiffness; *2*) BDM increased force enhancement and stiffness enhancement compared with the experiments performed in Ringer solution; and *3*) lowering the frequency of stimulation did not change force enhancement and stiffness enhancement compared with experiments performed at fused tetanic frequencies. In short, there was a direct relationship between enhanced force and stiffness, before and after changing the frequency of stimulation and adding BDM, suggesting that force enhancement is caused partially by an increase in the proportion of attached cross bridges after stretch.

Control experiments (Ringer solution). In this study, force enhancement after stretch was associated with an increase in fiber stiffness. Although this result is similar to what had been

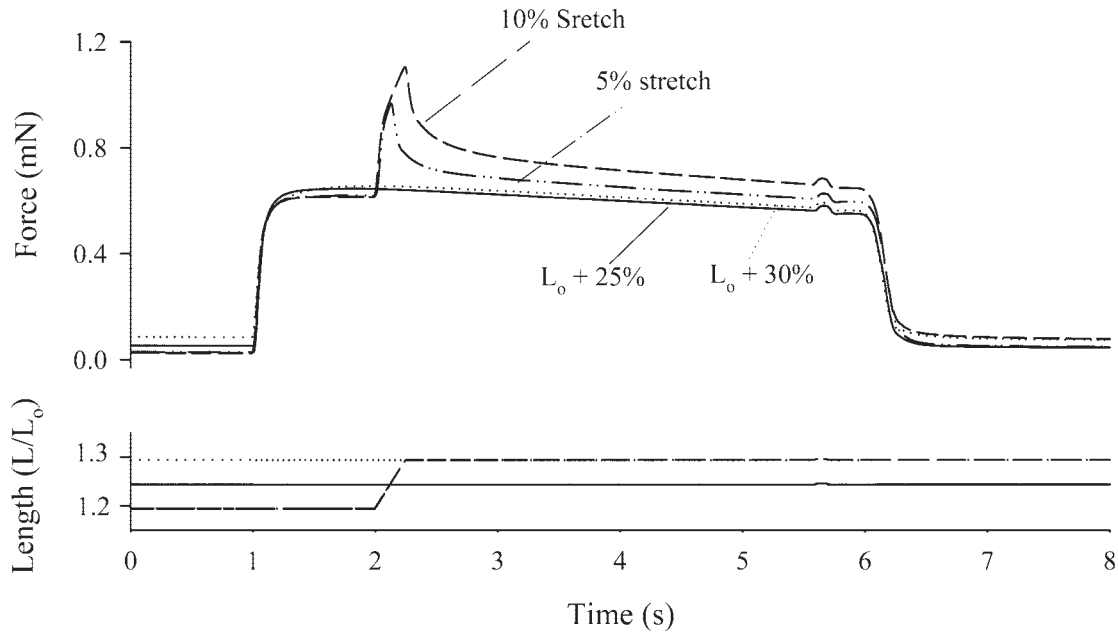

Fig. 3. Force-time histories of contractions in a single fiber activated in Ringer solution. Isometric reference contractions were produced at L_o + 25% and L_o + 30%, and active stretches of 5 and 10% fiber lengths ending at L_o + 25% and L_o + 30%, respectively, are also shown. Stretches were performed at a speed of 40% fiber length/s. After both stretches, the isometric steady-state force was greater than that produced during the corresponding isometric reference contractions. Force enhancement was greater after 10% compared with 5% stretches. Stiffness at isometric steady state after stretch was also greater than that measured during the corresponding isometric reference contractions. *L*, fiber length.

Table 2. *Total force produced during isometric contractions and after stretches of 5 and 10% fiber length*

Condition	Isometric Force L_o + 25%, mN	Force After Stretch L_o + 25%, mN	FE, mN	FE, %	Isometric Force L_o + 30%, mN	Force After Stretch L_o + 30%, mN	FE, mN	FE, %
Control	0.57±0.11	0.65±0.12	0.07±0.02	13.3±2.4*	0.58±0.12	0.68±0.13	0.10±0.02	17.9±5.5*
2 mM BDM	0.45±0.07	0.52±0.09	0.07±0.02	15.6±4.6†	0.47±0.09	0.60±0.10	0.12±0.02	27.2±7.5*†
5 mM BDM	0.30±0.09	0.36±0.09	0.06±0.01	20.4±5.1*†	0.28±0.09	0.45±0.12	0.17±0.03	68.1±20.6*†
50% Fr	0.52±0.10	0.58±0.11	0.06±0.01	12.6±3.4*	0.51±0.10	0.60±0.11	0.08±0.02	17.3±5.5*
25% Fr	0.40±0.08	0.45±0.10	0.05±0.03	12.4±6.5*	0.45±0.09	0.51±0.09	0.07±0.02	15.9±5.4*

Values are means ± SD. All stretches started at L_o + 20%. Force enhancement (FE) is given in absolute terms (mN) and relative to the isometric force produced at the corresponding length (%). *Stretch significantly different from isometric contraction, $P < 0.05$. †Force enhancement significantly different from control situation, $P < 0.05$.

observed previously in whole muscles (14), studies investigating stiffness after stretch in single fibers were inconclusive. On the one hand, Sugi and Tsuchiya (31) suggested that fiber stiffness in the force-enhanced state was similar to that observed during the isometric reference contractions (their Figs. 2 and 3). On the other hand, Julian and Morgan (18) observed that fiber stiffness was increased after stretch compared with the stiffness observed in the isometric phase preceding the stretch. The present results are the first to demonstrate convincingly an increased stiffness in the force-enhanced state compared with the stiffness in the corresponding isometric reference contractions.

Although our results of an increased fiber stiffness in the force-enhanced state agree with some published results (14), they do not agree with others (31), and it is not obvious why apparently similar experiments should give conflicting results. However, interpretation of stiffness results must be made with caution. Stiffness does not only reflect the proportion of attached cross bridges but also the cross-bridge attachment distribution (9, 10) and non-cross-bridge compliant structures present in the muscle or fiber preparation (2, 4, 9, 10). Furthermore, it is known that filaments' compliance increases during muscle activation and force generation, which would underestimate cross-bridge stiffness during our measurements. Since the relevant stiffness comparisons in the present study were made between isometric reference contractions [at a given length and for a given condition (activation, BDM)] and the corresponding isometric contraction after stretch, filaments compliance does not play a critical role in the interpretations of our results. If one assumes that cross-bridge distribution is similar in the force-enhanced and isometric reference state, then increased fiber stiffness reflects an increase in the proportion of attached cross bridges. Therefore, the results of this study suggest that force enhancement is caused, at least in part, by an increase in the proportion of attached cross bridges.

Effects of BDM. It has been shown that BDM suppresses both isometric force and stiffness (2, 3, 13, 17, 29, 30), but decreases stiffness significantly less than force, thereby increasing the stiffness-to-force ratio (29, 30). This finding was confirmed in the present study (Fig. 2). The effects of BDM on force production are less pronounced than those observed by Bagni et al. (2). Although it may be a result of different muscle preparations (lumbrical vs. tibialis anterior) and temperatures (9 vs. 14°C), the reasons why BDM depresses force less in our experiments are unclear. The small decrease in stiffness with increasing concentrations of BDM suggests that BDM decreases to some extent the proportion of attached cross bridges. However, force decreased substantially more than stiffness with increasing concentrations of BDM.

BDM is thought to bind to cross bridges and decrease the rate of P_i release, while accelerating the rate of ATP hydrolysis (13, 17). As a consequence, BDM-treated cross bridges are thought to attach rapidly to actin and remain proportionally longer in the pre- than the post-powerstroke state compared with untreated controls. Therefore, BDM is expected to increase the proportion of weakly bound vs. strongly bound cross bridges (29). Such weakly bound cross bridges would contribute to stiffness but not to force, a situation that agrees with the results observed in the present experiments.

Force enhancement and stiffness enhancement were increased after BDM administration. The increase in stiffness suggests that force enhancement in BDM fibers is attained with a relative increase in the proportion of cross bridges attached to actin. However, force enhancement was higher than stiffness enhancement, suggesting that not the entire force enhancement was caused by an increase in the proportion of attached cross

Table 3. *Total stiffness produced during isometric contractions and after stretches of 5 and 10% fiber length*

Condition	Isometric Stiffness L_o + 25% mN/mm	Stiffness After Stretch L_o + 25% mN/mm	SE, mN/mm	SE, %	Isometric Stiffness L_o + 30%, mN/mm	Stiffness After Stretch L_o + 30%, mN/mm	SE, mN/mm	SE, %
Control	5.85±1.11	6.77±1.53	0.92±0.52	9.4±6.9*	5.59±1.15	5.59±1.35	0.75±0.73	13.6±13.9*
2 mM BDM	7.14±1.45	8.15±2.00	1.01±0.84	13.9±11.7*	6.68±1.37	8.50±2.28	1.82±1.31	27.3±19.8*†
5 mM BDM	6.90±1.40	8.34±2.00	1.45±0.85	20.7±11.6*†	7.98±1.58	10.15±2.76	2.18±2.40	29.0±31.4*†
50% Fr	5.80±1.18	6.32±1.39	0.51±0.41	8.83±7.3*	5.59±1.15	6.46±1.43	0.87±0.56	15.7±10.9*

Values are means ± SD from 6 experiments. All stretches started at L_o + 20%. Stiffness enhancement (SE) is given in absolute terms (mN/mm) and relative to the isometric stiffness produced at the corresponding length (%). *Stretch significantly different from isometric contraction, $P < 0.05$. †Force enhancement significantly different from control situation, $P < 0.05$.

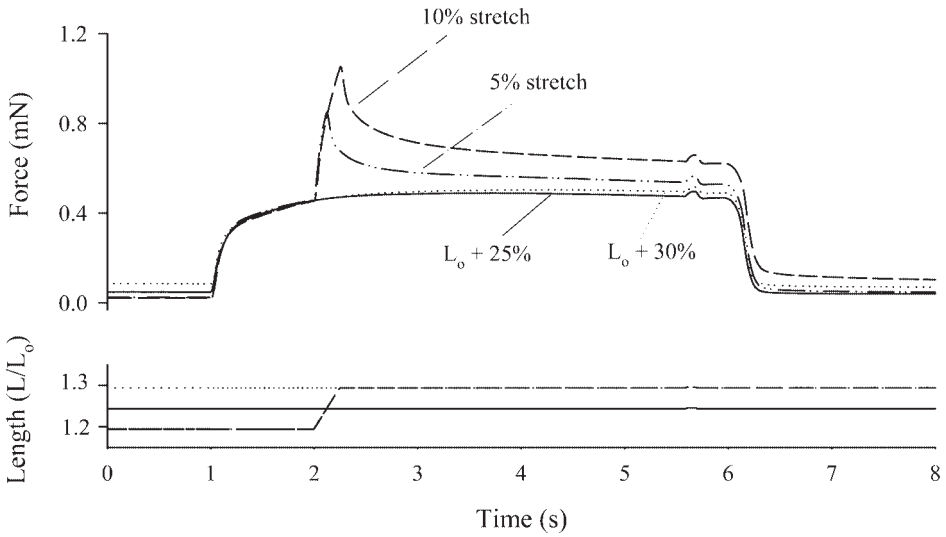

Fig. 4. Force-time histories of contractions in a single fiber after adding 2 mM 2,3-butanedione monoxime to the Ringer solution. Contractions are performed at the same lengths and mechanical conditions as those shown in Fig. 3, and force and stiffness are also increased after stretch.

bridges. Assuming that weakly bound cross bridges do not contribute significantly to force, combined with evidence that BDM slows the rate of P_i release (13), these results lead to the following hypothesis: force enhancement is partially caused by a stretch-induced increase in the proportion of attached cross bridges and a conversion of weakly bound to strongly bound (force producing) cross bridges. For a normal, reference fiber stretched in Ringer solution, the ratio of weakly bound to strongly bound cross bridges is low, and therefore the conversion from weakly bound to strongly bound cross bridges is expected to be small. In BDM-treated fibers, the ratio of weakly bound to strongly bound cross bridges is high. During stretch, weakly and strongly bound cross bridges contribute to stiffness and peak force attained at the end of the stretch. After stretch, a great number of the weakly bound cross bridges have been converted to force-producing, strongly bound cross bridges, thereby increasing force enhancement to levels above those observed in control fibers.

A series of studies that manipulate phosphate analogs and polyethylene glycol (6, 12) or changes in temperature (7, 24, 33), interventions that alter the proportion of weakly and strongly bound cross bridges, suggest that weakly bound cross bridges are involved in high forces produced during stretch. In here, we expand that hypothesis to propose that such conversion of weakly to strongly bound cross bridges can also be responsible, at least in part, for the long-lasting force enhancement observed after stretch.

Effects of stimulation rate. To further elucidate whether part of the force enhancement was caused by a shift of cross bridges from a weakly to a strongly bound state, we decreased fiber activation by lowering the frequency of stimulation. If force enhancement was partially caused by a conversion of weakly bound to strongly bound cross bridges, lowering the stimulation rate should cause levels of force enhancement similar to control experiments, and lower than BDM experiments, because there is no evidence that lowering the frequency of stimulation shifts cross bridges from strongly bound to weakly bound states. In fact, the level of force enhancement and stiffness during submaximal stimulation, after 5 and 10% stretches, was similar to that observed during high-frequency stimulation (Table 2).

Mechanisms of force enhancement. Although the results of this study, and previous studies performed in our laboratory (25), suggest that force enhancement is caused by changes in

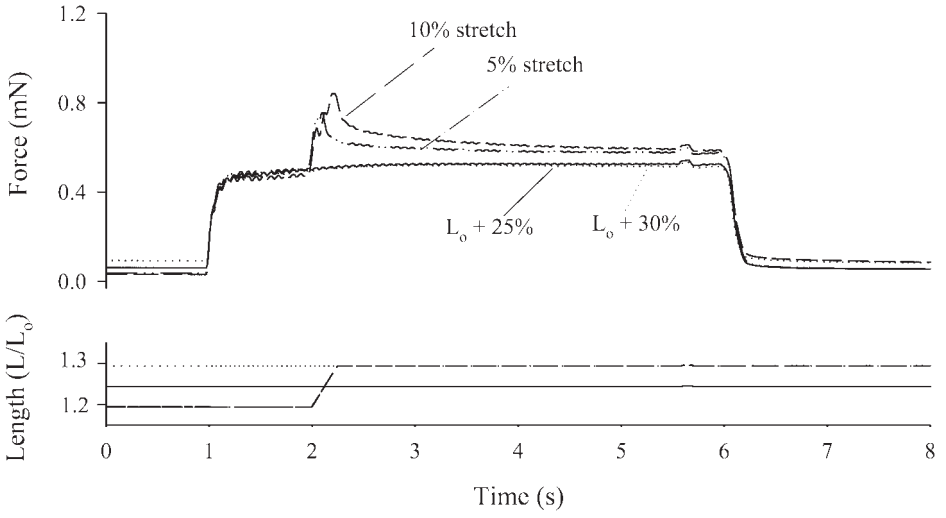

Fig. 5. Force-time histories of contractions in a single fiber activated in Ringer solution with 50% of maximal frequency of stimulation. Contractions were performed at the same lengths and mechanical conditions as those shown in Fig. 3, and force and stiffness are also increased after stretch.

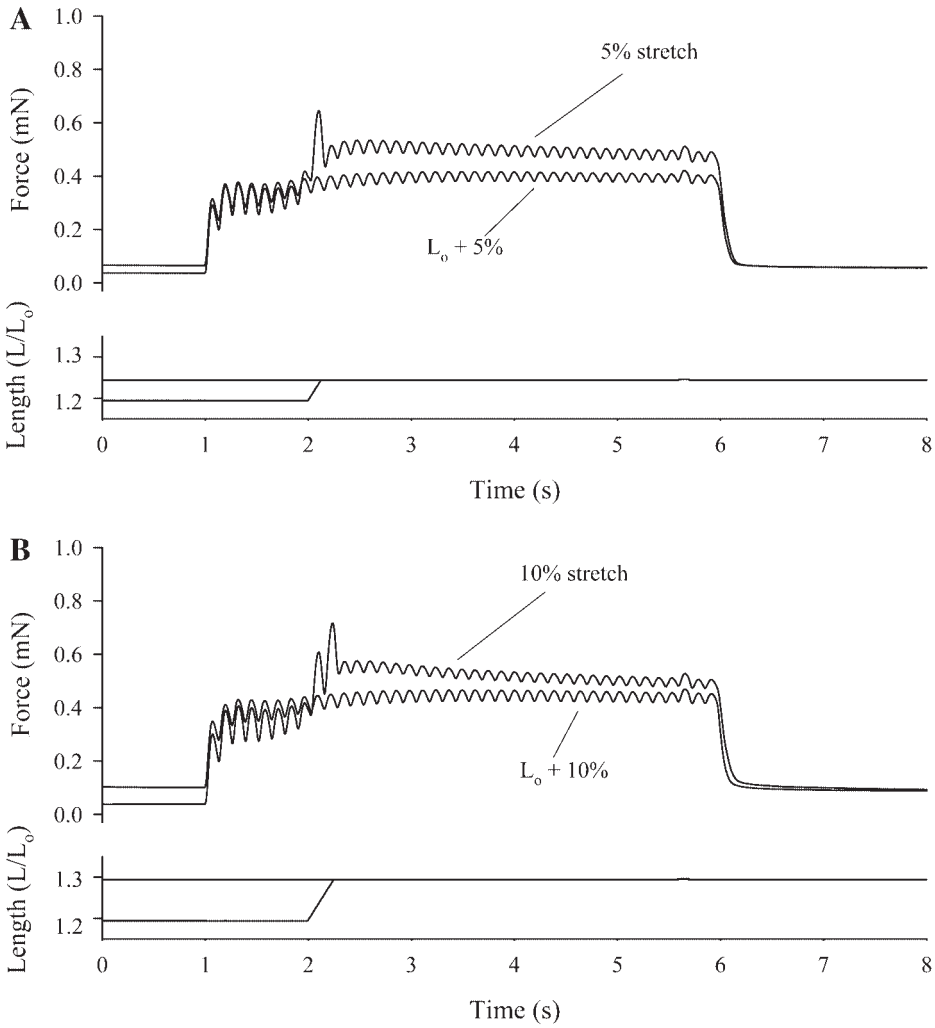

Fig. 6. Force-time histories of contractions in a single fiber activated in Ringer solution with 25% of maximal frequency of stimulation. *A*: isometric contraction was produced at L_o + 25%, and the active stretch of 5% fiber length was performed to L_o + 25%. *B*: isometric contraction was produced at L_o + 30%, and the active stretch of 10% fiber length was performed ending at L_o + 30%. Stretches were performed at a speed of 40% fiber length/s. In both cases, the isometric, steady-state force produced after stretch was greater than that produced during the corresponding isometric reference contractions.

the cross-bridge kinetics, there is evidence that force enhancement is associated with more than one mechanism (26). Two mechanisms that have been suggested in the literature are *1*) sarcomere length nonuniformity and instability (18, 21) and *2*) engagement of a passive element (15, 16, 25, 27).

With regard to *mechanism 1*, Julian and Morgan (18) observed that, during stretch on the descending limb of the force-length relationship, sarcomeres near the center region of muscle fibers stretched more than sarcomeres near the end of the fibers, which remained almost isometric. Morgan (21) suggested that force enhancement was produced by sarcomeres that did not stretch significantly, whereas the sarcomeres that stretched to lengths beyond myofilament overlap would be supported by passive elements, rendering an equilibrium force that would be greater than that produced during isometric contractions. Although there is evidence that this mechanism may work in some situations (5, 18, 32), there are results that are incompatible with the sarcomere length nonuniformity theory. For example, sarcomere length does not diverge during the force-enhanced state (8); force enhancement is observed on the ascending limb of the force-length relationship, a region considered mechanically stable (23); and force enhancement exceeds the isometric forces at the plateau of the force-length relationship (28). Therefore, sarcomere nonuniformity may be responsible for part of the force enhancement, but it cannot explain it completely.

With regard to *mechanism 2*, force enhancement may be associated with the engagement of a passive element on activation and stretch. Evidence for this mechanism comes from studies showing an increase in the passive force after active, but not passive, muscle stretch observed in different muscle preparations (15, 16, 25, 27). This increase in passive force enhancement is long lasting and increases with stretch magnitude and initial muscle length, and it is independent of the speed of stretch (15, 16), characteristics that are similar to those of the total force enhancement. It has been suggested that the structure responsible for the increase in passive force after stretch of an activated muscle is titin (15, 16, 19, 25). However, the passive force enhancement is always smaller than the total force enhancement, and it does not occur at short muscle lengths. Therefore, it cannot explain solely the stretch-induced force enhancement.

Summary. Our results show a strong relationship between the relative increase in force and the relative increase in stiffness after active stretch of muscle fibers. This relationship is preserved for different stretch amplitudes and changed cross-bridge kinetics. Therefore, we propose that part of the observed force enhancement is caused by an increase in the proportion of

attached cross bridges. However, for all conditions (control, BDM, decreased frequency of stimulation), force enhancement was greater than stiffness enhancement, suggesting that there must be another factor contributing to force enhancement. On the basis of the results of this study, we propose that this other factor might be a stretch-induced conversion of weakly bound to strongly bound cross bridges. This latter effect appears to be particularly dominant for the situation with the greatest stretch amplitude (10%) and highest (5 mM) BDM concentrations.

GRANTS

This study was funded by Canadian Institutes of Health Research (CIHR).

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