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Detection of Radial Crossbridge Force by Lattice Spacing Changes in Intact Single Muscle Fibers

G. CECCHI, M. A. BAGNI, P. J. GRIFFITHS,* C. C. ASHLEY, Y. MAEDA

Time-resolved lattice spacing changes were measured (10-millisecond time resolution) by x-ray diffraction of synchrotron radiation in single intact muscle fibers of the frog *Rana temporaria* undergoing electrically stimulated tension development during application of stretches and releases. Ramp releases, which decreased fiber length at constant speed, caused a lattice expansion. After the ramp, increasing tension during recovery was accompanied by lattice compression. Ramp stretches caused a compression of the lattice. While the fiber was held at a constant length after the stretch, tension decreased and lattice spacing increased. These observations demonstrate the existence of a previously undetected radial component of the force generated by a cycling crossbridge. At sarcomere lengths of 2.05 to 2.2 micrometers, the radial force compresses the myofilament lattice. Hence, the myofilament lattice does not maintain a constant volume during changes in force.

ACCORDING TO THE MOST WIDELY accepted theory of muscle contraction (*1*), tension development occurs as a result of the interaction of actin and myosin filaments along radial projections from the myosin filament (crossbridges). These projections contact the actin filament during activation and exert a force directed axially that is responsible for sarcomere shortening. However, this force may also have components directed radially with respect to the axis of the myosin filament. The action of such radial forces on the filament lattice depends on the geometrical arrangement of the components of the attached crossbridge structure. A compression of skinned fibers on entering the rigor state and on calcium activation (*2*) has been reported, but, until now, no such radial force component has been detected experimentally in intact fibers during electrically stimulated tension generation (tetanus). We report on time-resolved lattice spacing measurements performed on single intact skeletal muscle fibers of the frog during rapid length changes imposed on the fiber during tetani. Changes in the myofilament lattice during active tension development are consistent with the existence of a radially compressive force on the myofilament lattice associated with crossbridge activity at slack length. These results are inconsistent with constant volume theory and allow testing of particular theories of crossbridge force generation. In particular, they impose important constraints on the geometry of the structural change accompanying tension development by the crossbridge and suggest the importance of accounting for radial forces in current crossbridge theories.

During tetani, single muscle fibers were exposed to x-ray synchrotron radiation, and the equatorial x-ray diffraction pattern of the fiber was recorded. At the tetanus plateau, ramp shortening of a fiber at a velocity sufficient to reduce tension from its isometric level (P_0) to 0.05 P_0 caused a rapid shift in the position of the equatorial reflections toward the center of the diffraction pattern, which corresponds to an expansion of the myofilament lattice (*3*) and a partial reversal of the equatorial intensity changes associated with activation of the muscle toward the relaxed intensity pattern. While force remained low (because of the continuing shortening of the preparation), the lattice remained expanded. When shortening ceased, the fiber recovered tension rapidly to the new plateau level. The recovery of tension was associated with a compression of the lattice. An example of this behavior is shown in Fig. 1A. Although the expansion of the lattice associated with the shortening of the preparation is accompanied by a decrease in sarcomere length, the compression of the lattice during the recovery of tension occurs under virtually isometric conditions (Fig. 1B).

If the activated fiber behaves as a constant volume system as already reported for passive fibers (*4*), then sarcomere shortening would be expected to result in a lattice expansion, being apparently consistent with the present observation. However, lattice expansion is bigger than expected for constant volume behavior. In fact, calculation of lattice volume from the lattice spacing and the sarcomere length changes shows that a considerable increase in lattice volume occurred during shortening (Fig. 1C). During the subsequent recovery of tension after the release was terminated, the lattice volume returned to a value close to that found before the release.

During a ramp stretch, force increased rapidly at first, after which it stabilized at a new level, and the remainder of the stretch ramp occurred under isotonic conditions (Fig. 2, A and B). Lattice spacing was reduced during the rise of force and underwent reextension after completion of the stretch, during the return of force to the isometric level. By plotting the lattice volume calculated from the sarcomere length signal and the lattice spacing, we found that this behavior cannot be accounted for if constant volume of the lattice is assumed (Fig. 2C).

Our findings show that, during shortening of a fiber at a rate sufficient to reduce force to 0.05 P_0, a lattice expansion occurs. However, the time course of this expansion is more similar to the time course of the fall of force and is not similar to the ramp length change as would be expected from constant volume behavior. During the recovery of tension after completion of a ramp, virtually no change in sarcomere length was observed. Since during this period tension is rising, the expected behavior, according to constant volume constraints, should be a lattice expansion as series compliance is reextended and the contractile system shortens (*5*). However, what is actually observed is a compression of the lattice. A similar argu-

G. Cecchi and M. A. Bagni Dipartmento di Scienze Fisiologiche, Universita degli studi di Firenze, I-50134 Florence, Italy.
P. J. Griffiths and C. C. Ashley, University Laboratory of Physiology, University of Oxford, OX1 3PT, United Kingdom.
Y. Maeda, European Molecular Biology Laboratory Outstation, Deutsches Elektronen Synchrotron, Hamburg D2000, Federal Republic of Germany.

*To whom correspondence should be addressed.

ment can be applied to the recovery from a stretch. The overall behavior of the lattice spacing is therefore in conflict with the predictions of the constant volume theory. The time course of the lattice spacing change accompanying force recovery is rather slower than force recovery; however, the force and lattice spacing recovery rates are related to one another. This can be seen in Figs. 1 and 2, where the quicker force recovery after a stretch is accompanied by a quicker lattice spacing expansion.

The data would be well accounted for if the development of isometric tension were associated with a compressive radial force on the myofilament lattice. This would explain both lattice compression and expansion during the recovery of force after release and stretch. It would also account for the volume changes occurring during the applied length ramps where, for example, expansion during a release would be caused not merely by fiber shortening but also by force reduction, and hence a discharge of the radial compression caused by the crossbridges. Compression of the lattice by the force-generating process is the opposite effect to that predicted if force development occurred by electrostatic repulsion of the myosin filaments. In the conventional crossbridge model of force development, longitudinal tension development may be accompanied by either a compression or an expansion of the lattice, depending on the geometrical arrangement of the components of the crossbridge (*6*). Our findings permit us to reject those models that predict a lattice expansion at a sarcomere length of 2.2 μm. However, it could be suggested that the compression of the lattice during the recovery from release or the expansion accompanying recovery from stretch represents some crossbridge-independent aspect of muscle behavior. To test this possibility, we performed ramp stretches followed by ramp releases on unstimulated fibers. The recovery phase of lattice spacing after the completion of the ramps was eliminated. We conclude that the recovery phase in active muscle is associated with the development of tension by the fiber, and hence with crossbridge activity (*5*).

If the fiber volume were to change by the same amount as the change in lattice volume that we have shown here, the radial forces required would be unacceptably large (*6*). However, if the proteins associated with the myofilament lattice make only a small contribution to the osmotic strength of the intracellular medium, then compression of the lattice could cause expulsion of fluid from the lattice into the intermyofilament space without development of large osmotic pressure differences between the filament lattice and the surrounding intermyofibrillar fluid, and without a change in total fiber volume. At the molecular level, the changes in lattice spacing that result from tension development are appreciable. The length change required to just discharge longitudinal isometric tension developed by an individual crossbridge (γ_0) is thought to be of the order of 4 nm. From our findings, the expected shift of radial separation of adjacent actin and myosin filaments during the discharge of isometric tension would be about 0.25 γ_0, that is, of comparable size. Although one is a radial displacement and the other is longitudinal, both may be components of a diagonal force generated by a cycling crossbridge. If this shift also occurs in the submillisecond time scale of the tran-

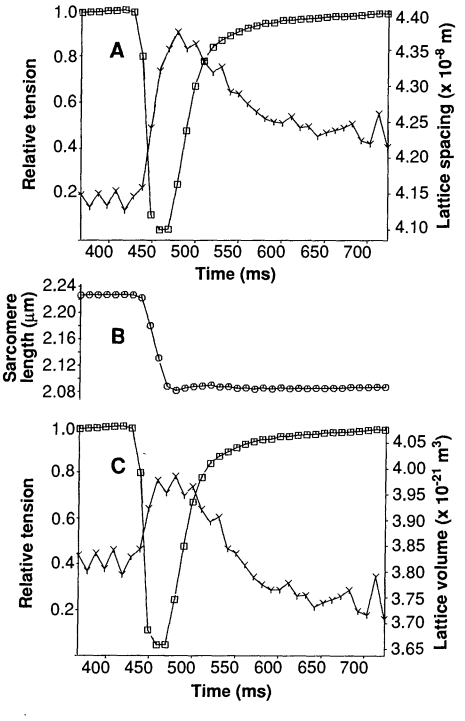

Fig. 1. (**A**) Lattice spacing (Y) and tension (□), (**B**) sarcomere length (○), and (**C**) lattice volume (Y) and tension (□) during and subsequent to a ramp release. All experiments were conducted at the Deutsches Elektronen Synchrotron facility (DESY) in Hamburg. A fuller description of the experimental procedures and methods will be given elsewhere (*8*). Intact single muscle fibers were isolated from the tibialis anterior muscles of *Rana temporaria* and mounted in the experimental chamber. Aluminum clips were attached to the tendons to provide mechanical connection to a capacitance force transducer (resonance frequency, 50 kHz) and a length step generator capable of a 500-μm release or stretch. Sarcomere length was adjusted to between 2.05 and 2.2 (±0.01) μm. Fibers were stimulated along their whole length by platinum wire electrodes with brief pulses (0.5-ms duration) at a frequency of 20 Hz for 0.6 s to induce tetani. During tetani, fibers were exposed to a beam of synchrotron x-ray radiation (beam dimensions, 4 mm by 300 μm), and the resulting equatorial x-ray diffraction pattern was detected by a one-dimensional crossed-wire detector (*9*). Simultaneously the fibers were exposed to a HeNe laser beam (collimated to a 250-μm diameter spot) in order to measure time-resolved sarcomere length changes from the resulting laser diffraction pattern with a laser diffractometer designed for use at the x-ray beamline. The lattice spacing (*3*) was calculated from the 50% point of integrated intensity of the equatorial reflections (10 and 11) after background scatter had been subtracted. Equatorial patterns were collected in 10-ms sampling periods during 400 ms of exposure to synchrotron radiation. The resulting equatorial diffraction patterns were then averaged over up to 90 tetani to improve definition of the individual reflections. All lattice dimensions are in nanometers. Lattice volume was calculated as the square of the spacing times sarcomere length. Isometric tension, 334 kN m^{-2}. Velocity of release, 2.2 muscle lengths per second. Temperature, 4°C.

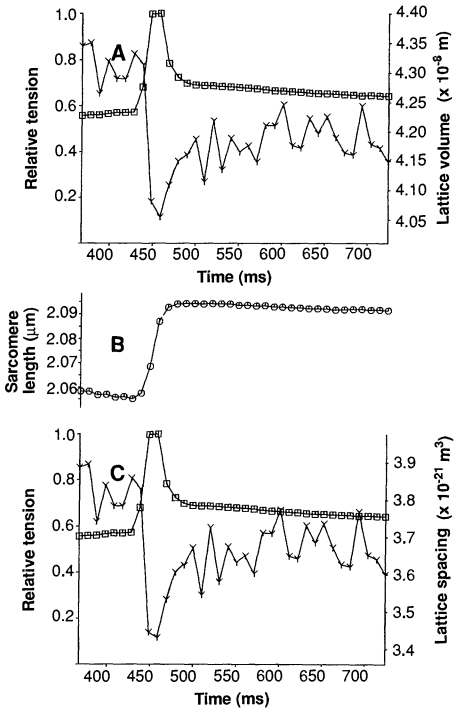

Fig. 2. Same as in Fig. 1 but for a quick stretch. Isometric tension, 301 kN m^{-2}. Velocity of stretch, 2.2 muscle lengths per second. The elevated tension level after the stretch is completed is a well-known phenomenon and is described elsewhere (*10*). Temperature, 4°C. Both Figs. 1 and 2 are plots of individual fibers representative of a group of similar experiments. For the shortening experiments (Fig. 1) 22 fibers were used; for the stretch experiments, 5 fibers. The mean compression of the lattice during recovery from a release sufficient to discharge isometric tension was 1.218 ± 0.387 nm, mean expansion during recovery from a stretch that increased tension to 1.84 times isometric tension was 1.059 ± 0.527 nm. A rough estimate of the radial force per myosin filament may be obtained by comparison with the osmotic force required to compress the lattice in relaxed skinned fibers, given by Matsubara *et al.* (*2*). Taking the mean lattice spacing at slack length as 41.8 nm, we find that the calculated radial force needed to induce a 1.218-nm lattice compression would be 1.18 × 10^{-10} N, or ~23% of the longitudinal force.

sients associated with the recovery of tension from a quick release, then it may have a bearing on the interpretation of such transients described by some models of cross-bridge behavior (7).

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Inhibition of HIV-1 Replication by a Nonnucleoside Reverse Transcriptase Inhibitor

VINCENT J. MERLUZZI,* KARL D. HARGRAVE, MARK LABADIA, KARL GROZINGER, MARK SKOOG, JOSEPH C. WU, CHENG-KON SHIH, KRISTINE ECKNER, SUSAN HATTOX, JULIAN ADAMS, ALAN S. ROSEHTHAL, RONALD FAANES, ROBERT J. ECKNER, RICHARD A. KOUP, JOHN L. SULLIVAN

A series of dipyridodiazepinones have been shown to be potent inhibitors of human immunodeficiency virus–1 (HIV-1) reverse transcriptase (RT). One compound, BI-RG-587, had a K_i of 200 nanomolar for inhibition of HIV-1 RT that was noncompetitive with respect to deoxyguanosine triphosphate. BI-RG-587 was specific for HIV-1 RT, having no effect on feline and simian RT or any mammalian DNA polymerases. BI-RG-587 inhibited HIV-1 replication in vitro as demonstrated by in situ hybridization, inhibition of protein p24 production, and the lack of syncytia formation in cultured human T cell lines and freshly isolated human peripheral blood lymphocytes. Cytotoxicity studies of BI-RG-587 on human cells showed a high therapeutic index (>8000) in culture.

THE REVERSE TRANSCRIPTASE (RT) of HIV-1 is required for early proviral DNA synthesis and is thus a prime target for antiviral therapy against acquired immunodeficiency syndrome (AIDS) (*1*). Inhibition of the RT-catalyzed polymerization of DNA from viral RNA inhibits virus replication. In most cases, effective inhibitors are nucleoside analogs that are converted to triphosphates by cellular enzymes and act as chain terminators (*2, 3*). The first approved drug for use in HIV-1 infection was the nucleoside analog azidothymidine (AZT) (*4*). Although this compound has been shown to be of benefit in HIV-1–infected individuals, there are toxic side effects associated with its use (*5*), and complete inhibition of viral replication is usually not achieved (*6*). In addition, emergence of AZT-resistant strains may complicate its use in long-term therapy (*7*).

From a previous program involving the synthesis of muscarinic receptor antagonists, we identified a series of dipyridodiazepinone inhibitors of HIV-1 RT polymerase. Compounds were optimized on the basis of potency against HIV-1 RT with a favorable pharmacokinetic profile and lack of ancillary pharmacologic activities. The compound BI-RG-587 is a potent inhibitor of HIV-1 RT and does not have muscarinic or benzodiazepine (peripheral and central) activities (*8*). Further analysis has shown similar potency of BI-RG-587 on the inhibition of HIV-1 in cell culture. Since BI-RG-587 is not a nucleoside analog structure, it is hoped that the clinical side effects observed in AIDS patients treated with nucleoside-based chain terminators of RT such as AZT and 2′,3′-dideoxyinosine (ddI) will not be observed.

BI-RG-587 was synthesized as described in the legend to Fig. 1. The K_i value for RT inhibition by BI-RG-587 was 200 nM, and inhibition was noncompetitive with respect to deoxyguanosine triphosphate (dGTP) (Fig. 2). The noncompetitive character of this inhibition was consistent with, but not proof for, an allosteric binding site on the binary (RT:template-primer) or ternary (RT:template-primer:dGTP) complex of the enzyme. Template-primer binds before deoxynucleotide (*9*), therefore a compound that binds when dGTP is bound apparently occupies a site distinct from the template-primer site. In addition, BI-RG-587 inhibits RT regardless of whether RT was assayed with poly(rA):oligo(dT) [median inhibition concentration (IC_{50}) = 100 nM] or a het-

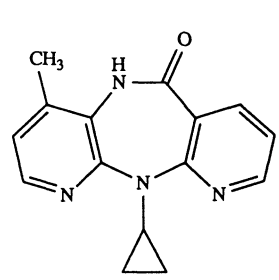

Fig. 1. BI-RG-587. BI-RG-587 was synthesized in four steps starting from 2-chloro-4-methyl-3-nitropyridine. This was reduced to 3-amino-2-chloro-4-methylpyridine, which was then condensed with 2-chloronicotinic acid chloride to form the amide. Reaction with cyclopropylamine followed by cyclization provided the desired compound, BI-RG-587.

V. J. Merluzzi, K. D. Hargrave, M. Labadia, K. Grozinger, M. Skoog, J. C. Wu, C.-K. Shih, K. Eckner, S. Hattox, J. Adams, A. S. Rosenthal, R. Faanes, R. J. Eckner, Boehringer Ingelheim Pharmaceuticals, Inc., Ridgefield, CT 06877.
R. A. Koup and J. L. Sullivan, Department of Pediatrics and Medicine, Program in Molecular Medicine, University of Massachusetts Medical School, Worcester, MA 01605.

*To whom correspondence should be addressed.