Cross-Bridge Kinetics of Rabbit Single Extraocular and Limb Muscle Fibers

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PURPOSE. To gain insights into the functional significance of myosin heavy-chain (MyHC) heterogeneity by comparing the mechanical kinetic properties of single rabbit extraocular muscle (EOM) fibers with those of limb fibers. EOMs are known to contain developmental and EOM-specific MyHCs in addition to those present in limb muscles, and MyHCs profoundly influence muscle mechanics.

METHODS. Isometric cross-bridge kinetics were analyzed in Ca2+-activated single glycerinated fibers from rabbit EOM and limb fast and slow muscles at 15°C by means of mechanical perturbation analysis. The plots of stiffness and phase against frequency display a characteristic frequency, \( f_{\text{min}} \), at which stiffness is minimum, and phase shift is zero. The value of \( f_{\text{min}} \) is independent of Ca\(^{2+}\) or force level but reflects the kinetics of cross-bridge cycling.

RESULTS. Analysis of 121 limb fast fibers gave \( f_{\text{min}} \) values ranging from 10 to 26 Hz. \( f_{\text{min}} \) for the 10 slow soleus fibers was 0.5 Hz. Analysis of 170 EOM fibers gave \( f_{\text{min}} \) values in the range for fast limb fibers, but in addition yielded \( f_{\text{min}} \) values below (4–9 Hz) and above (27–33 Hz) this range.

CONCLUSIONS. The wider range of mechanical kinetic characteristics in EOM fibers compared with limb fibers is likely due to the expression of developmental (low \( f_{\text{min}} \)) and EOM-specific (high \( f_{\text{min}} \)) MyHCs in addition to isoforms present in adult limb muscles. The considerable diversity of functional characteristics in EOM fibers is likely to be important for rotating the eyeball at various speeds during tracking and for executing saccades over a wide range of angles. (Invest Ophthalmol Vis Sci. 2000;41:3770–3774)

EXTRAOCULAR MUSCLES (EOMs) have a diverse repertoire of functions including steady eyeball fixation, slow vergence movements, pursuit movements at various speeds, and high-speed saccades over a wide range of angles.1 These functional intricacies are reflected in the complex fiber types seen in EOMs. EOM fibers have been classified into six types on the basis of histochemistry, ultrastructure, and innervation.2 This system of classification differs markedly from that used to classify functionally different limb muscle fibers, the latter subserving locomotion and posture. Limb muscle fibers are classified into four types: type I (or slow) and three subtypes (IIA, IIX, IIB) of type II or fast fibers, each expressing a different isoform of myosin heavy chain (MyHC).3 An indicator of the complexity of EOMs is seen in the fact that nine different myosin heavy chains (MyHCs) have been identified in these muscles in adult animals. These include isoforms found in adult limb fast (IIA, IIX, IIB-MyHC) and slow (type I-MyHC, synonymous with cardiac \( \beta \)-MyHC) fibers,4,5 but in addition, MyHCs found in developing (embryonic- and fetal/neonatal-MyHCs) but not in mature limb muscles.4,5 the cardiac-specific \( \alpha \)-MyHC6–7 as well as two EO-specific isoforms: the EO-MyHC8,9 and the slow-tonic MyHC.10

Through its cyclic interaction with actin during muscle contraction, myosin controls the kinetics of energy transduction from ATP into mechanical work. In limb muscle fibers, the speed of contraction and thus the power and efficiency of muscle fibers are controlled principally by the type of MyHC.11 The complexity of MyHC types found in EOMs suggests that mechanical properties of single fibers in these muscle should be correspondingly complex. There is little information in the literature on mechanical properties of single EOM fibers. Published works on contractile properties of EOMs are limited to analyses of isometric and isotonic contractile characteristics of whole EOMs.12 These show that isometric contraction times are very short while the twitch:tetanus tension ratio is low, compared with fast limb muscles of the same species. The maximal speed of shortening (\( V_{\text{max}} \)) is generally higher than that of the fastest limb muscle in the same animal. For the rabbit, \( V_{\text{max}} \) of EOM is 42% higher than that of the extensor digitorum longus, a limb fast muscle.12 Whole muscle mechanical data give little insight into the functional significance of MyHC complexity. Single-fiber analysis of EOM has been limited to the measurement of isometric force at various Ca\(^{2+}\) and Sr\(^{2+}\) concentrations.13 Studies on cross-bridge cycling kinetics have not been reported for single EOM fibers.

The mechanical characteristics of single muscle fibers can be analyzed using low amplitude length oscillations at various frequencies to probe the dynamic stiffness of active fibers.14 This analysis yields a parameter, \( f_{\text{min}} \), the frequency at which dynamic stiffness of the fiber is a minimum. The value of this parameter reflects the kinetics of cross-bridge cycling in the

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in this article, we compared the mechanical characteristics of single rabbit EOM fibers with those of limb fibers using this method, to gain insights into the functional significance of MyHC heterogeneity of EOMs.

Methods

Muscle Fiber Preparation

Muscle fibers from four adult female and two male New Zealand White rabbits were used in these experiments. The use of these animals was approved by the Animal Ethics Committee of our institution and adhered to the ARVO Statement for use of animals. The animals were killed by sodium pentobarbitone overdose. Muscle bundles were dissected from EOMs, vastus lateralis (IIA, IIX, IIB fibers), extensor digitorum longus (predominantly 2A, 2X fibers), and soleus (slow fibers) muscles. The dissected muscle bundles were pinned out on a flat wooden stick and kept in skinning solution and agitated continuously for 24 hours at 4°C. After 24 hours the muscles were placed in storage solution and kept at −20°C and used within 4 weeks.

Skinning solution contained (in mM): 5 MgCl₂, 60 HEPES (pH 6.7), 108 Na acetate, 5 NaH₂PO₄, 2.5 EGTA (stock solution adjusted with NaOH to pH 7), 0.01% (g/ml) NaN₃, 0.01% (g/ml) DTT, 50% (v/v) glycerol.

Storage solution contained (in mM): 5 MgCl₂, 60 HEPES (pH 6.7), 108 K acetate, 5 K₂HPO₄, 2.5 EGTA (stock solution adjusted with KOH to pH 7), 0.01% (g/ml) NaN₃, 0.01% (g/ml) DTT, 50% (v/v) glycerol.

Mechanical Measurements

Both relaxing and activation solutions contained (in mM): 7 EGTA (stock solution adjusted with KOH to pH 7.0), 5.26 MgCl₂, 20 imidazole, 5 K₂HPO₄, 5 ATP, 20 creatine phosphate. In addition, activation solution contained 7.36 mM CaCl₂. Because of the relatively short shelf life of creatine phosphokinase, it was kept at −20°C and added directly to the activation solution in the fiber bath as required (1 mg/ml). The pH of both solutions was adjusted to 7.0 by adding either KOH or HCl at room temperature. The ionic strength of the solution was determined by the algorithm of Perrin and Sayce. KCl was used to bring the ionic strength to the required level. In this study, the ionic strength was 173 mM in relaxing solution and 168 mM in activation solution. Both solutions were stored at −20°C.

Experimental Set-up and Procedure

Single muscle fibers, typically 2 to 3 mm in length, were randomly teased from small glycerinated bundles at 0°C by means of jeweler’s forceps. One end of the fiber was glued onto a length driver (P-841.10; Physik Instruments, Waldbronn, Germany). The fiber was viewed through an inverted microscope (Fluorovert; Leitz, Wetzlar, Germany) at a magnification of ×400, and the sarcomere length of the fiber was adjusted to 2.5 μm by means of a calibrated eyepiece micrometer. The temperature of the fiber bath solution was maintained by means of a peltier module (KSM-0617; Komatsu Electronics, Tokyo, Japan) and a temperature sensor (AD 590; Analog Devices, Norwood, MA), which provided a feedback signal for a custom-built proportional-integral controller. A digital thermometer (KM-330; Kane Instruments, Bedford, MA) was located in the fiber bath solution to provide an independent and continued read-out of temperature.

Fiber length was perturbed by a signal that was software-generated and introduced to the fiber via D/A conversion and the length driver. The form of the signal was pseudorandom binary noise (PRBN), which enabled the calculation of stiffness and phase spectra with greater resolution and in less time compared with the sinusoidal technique. The amplitude of the length signal was typically 0.05% of the fiber length.

Single fibers were incubated for approximately 5 minutes in the muscle bath filled with relaxing solution. Activation of the fiber was achieved by changing from the well with relaxing solution to one containing activation solution. The Ca²⁺ concentration of activation solution was pCa 4.0, which ensured maximal activation of the fiber. The temperature of the bath solution was set at 15°C. When steady tension had been attained, fiber length was perturbed with the PRBN signal. The length changes together with the resulting force responses of the fiber were sampled by the control computer via A/D conversion. Fast Fourier transforms of the length and force data yielded the stiffness and phase values. The stiffness and phase data were smoothed, using a three-point convolution procedure, and displayed on a digital plotter (7225A; Hewlett-Packard, Palo Alto, CA). fₘᵢₙ was evaluated from the dynamic stiffness plots by noting the frequency at which stiffness was at the minimum.

Results

The dynamic stiffness and phase plots of single fibers from EOM displayed the characteristic minimum in stiffness and the accompanying mini-max feature in the phase curve. Figure 1 show respectively the dynamic stiffness (Fig. 1A) and phase (Fig. 1B) plots of two EOM single fibers whose fₘᵢₙ values were located at the two extremes of the range for the 170 fibers investigated. For comparison, Figures 1C and 1D shows stiff-
approximately 5% of extraocular fiber investigated in this study showed no minimum in their dynamic stiffness at 0.1 Hz or higher.

**DISCUSSION**

**Frequency Domain Analysis of Muscle Mechanics**

The use of frequency domain analysis of muscle mechanics was introduced as an alternative to classical force:velocity measurements for characterizing muscle mechanics in dealing with insect flight muscles, which are intrinsically oscillatory. This approach is particularly advantageous for insect flight muscles because their sarcomeres are unable to undergo the long-range sliding characteristic of vertebrate striated muscles necessary for force:velocity measurements. The frequency domain method for characterizing isometric cross-bridge mechanics was found to be generally applicable to vertebrate skeletal and cardiac muscles.

In the original method of analyzing the dynamic stiffness of contracting muscle, sinusoidal length changes over a range of frequencies were applied to the muscle sequentially, and the resulting near sinusoidal force and phase changes at each frequency were used to derive the dynamic stiffness characteristics. These dynamic stiffness characteristics can be related to the time courses of tension transients in responses to rectangular changes of muscle length. For very small amplitudes, these two methodologies are approximately related through the Fourier transform. In terms of this relationship, it can be deduced that $1/f_{\text{min}}$ correlates with the time $t_2$ taken to complete Phase 2 of such a tension step transient.

The method used in this article differed from the classical method in that the applied oscillations of muscle length took the form of PRBN. This signal has encoded within itself the full range of frequencies of interest. Fourier analysis of the PRBN length oscillations and the resulting interrupted tension transients were used to derive the dynamic stiffness values. The advantages of this method are that it gives a high-resolution plot of the data as a function of frequency and reduces the data acquisition time. The method has been shown to give comparable results to the classical sinusoidal method.

**Significance of $f_{\text{min}}$**

It is well established that dynamic stiffness and phase parameters of contracting muscle reflect cross-bridge cycling rates. In a three-state (detached, attached but not force-generating, attached and force-generating), cross-bridge model, $f_{\text{min}}$ is sensitive to rate constants for the power-stroke and the cross-bridge detachment rate during isometric contraction. At 25°C, $f_{\text{min}}$ values range from 60 Hz for insect flight muscles to 1 to 2 Hz for mammalian cardiac muscle. The value is correlated with the MyHC structure and ATPase activity of the myosin. This is well illustrated in rat cardiac muscle. Rat ventricular muscle may contain V1 or V3 myosin, depending on the thyroid state. V1 is composed of two α-MyHCs and has a high ATPase activity, whereas V3 myosin is composed of two β-MyHCs and has low ATPase activity. The ratio of $f_{\text{min}}$ values for V1 and V3 is 2:1, the same as the ratio of their myosin ATPase activities and $V_{\text{max}}$. $f_{\text{min}}$ is independent of the level of muscle force but is sensitive to the temperature at which measurements are made.

The correlation between complex stiffness and tension transients is further supported from simulation studies, where...
were wider than that of limb fast fibers. In addition to fast muscles with the fetal isoforms with the tension step transient parameter $f_{min}$ having the same MyHC. 27

Because IIB fibers have the highest and IIA fibers the lowest $f_{min}$ values between 10 and 17 Hz, whereas 2B fibers have values between 19 and 26 Hz. The correlation of $f_{min}$ with MyHC isoforms is further supported by the correlation of MyHC isoforms with the tension step transient parameter $t_2$. 29–31

Because IIB fibers have the highest and IIA fibers the lowest $V_{max}$ values, there is a broad correlation between $f_{min}$ and $V_{max}$ in skeletal muscle fibers, as found for cardiac muscle discussed above.

$\textbf{f_{min} Values of Limb Muscle Fibers}$

In this study, the distributions of $f_{min}$ values in 170 single fibers were wider than that of limb fast fibers. In addition to $f_{min}$ values in the range 10 to 26 Hz found in limb fast fibers, there were $f_{min}$ values below (4–9 Hz) and above this range (27–36 Hz). Values within the range found for limb fast muscle fibers are most likely due to the presence of fibers containing 2A, 2X, and 2B MyHCs. Coexpression of multiple isoforms of limb MyHC in EOM fibers is likely to produce only $f_{min}$ values within the range found in limb fibers. Values above and below this range found in EOM fibers are likely due to MyHCs found in EOM fibers only, namely, the developmental and EOM-specific isoforms. Although myosin light chains and other myofilament protein are able to affect mechanical properties of muscle fibers, rabbit EOMs are known to express fast isoforms of light chains, which are indistinguishable from those in limb fast muscle.32 Rabbit EOMs also express the same isoforms of tropomyosin and troponin T as limb muscle fibers, though the dominant isoform of TnT is TnT2, which is a minor component in limb fast fibers.33

During early postnatal life, limb muscles express embryonic and fetal/neonatal myosins. These myosins are progressively replaced by the appropriate MyHCs during the first few weeks of life.34,35 Functionally, all limb muscles in newborn animals are slow contracting. During the first few weeks of postnatal life, the developing fast muscle increases in speed of shortening by a factor or 2 to 3, whereas the speed of shortening of slow muscles remain unchanged.36,37 The developmental isoforms of MyHC are therefore associated with slower speed of contraction relative to fast isoforms. We suggest that $f_{min}$ values of 4 to 9 Hz are due to the persistence of embryonic and fetal/neonatal isoforms of MyHC in adult EOM fibers.

The $V_{max}$ of rabbit EOM is 42% faster than limb fast muscle, and this has been attributed to the presence of EOM MyHC. 12 In view of the correlation between $f_{min}$ and $V_{max}$, we may attribute the presence of $f_{min}$ values above 26 Hz to the presence of this EOM-MyHC.

We found that 5% of EOM fibers did not display a stiffness minimum in the range of 0.1 to 100 Hz. These fibers are probably slow-tonic fibers with very slow cross-bridge kinetics.

**Functional Significance of MyHC Complexity in EOMs**

Our results show that single EOM fibers have an extremely wide range of mechanical characteristics. This result represents a considerable advance in our understanding of EOM mechanics. The force:velocity relation of rat EOM is complex, deviating from the classical Hill’s hyperbolic relation, but can be fitted well by an exponential function with three constants.38 In the light of the current work, it is likely that the complexity of whole EOM mechanics is due to fibers with different intrinsic speeds contracting in parallel.

The diversity of $f_{min}$ values of single EOM fibers is no doubt generated by the expression in single fibers of myosins with different kinetic properties. Clearly, to provide the means of generating such functional diversity is likely to be the explanation for the well known but puzzling complexity of MyHC composition of EOMs. Presumably such functional diversity is found also at the motor unit level. The oculomotor system would thus have at its disposal motor units with a diverse range of speeds and thus have the potential for recruiting appropriate motor units for the task at hand. We suggest that for tracking movements at various angular speeds, motor units of matching speeds are recruited. The work involved in rotating the eyeball during a saccade will differ depending on the angle through which the eyeball has to rotate. We suggest that slower units are recruited for low-angle saccades, whereas faster and thus more powerful units are recruited for wide-angle saccades.

**References**


