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Effect of muscle length on cross-bridge kinetics in intact cardiac trabeculae at body temperature

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Dynamic force generation in cardiac muscle, which determines cardiac pumping activity, depends on both the number of sarcomeric cross-bridges and on their cycling kinetics. The Frank–Starling mechanism dictates that cardiac force development increases with increasing cardiac muscle length (corresponding to increased ventricular volume). It is, however, unclear to what extent this increase in cardiac muscle length affects the rate of cross-bridge cycling. Previous studies using permeabilized cardiac preparations, sub-physiological temperatures, or both have obtained conflicting results. Here, we developed a protocol that allowed us to reliably and reproducibly measure the rate of tension redevelopment (k_{tr}; which depends on the rate of cross-bridge cycling) in intact trabeculae at body temperature. Using K^+ contractures to induce a tonic level of force, we showed the k_{tr} was slower in rabbit muscle (which contains predominantly β myosin) than in rat muscle (which contains predominantly α myosin). Analyses of k_{tr} in rat muscle at optimal length (L_{opt}) and 90% of optimal length (L_{90}) revealed that k_{tr} was significantly slower at L_{opt} (27.7 ± 3.3 and 27.8 ± 3.0 s^{-1} in duplicate analyses) than at L_{90} (45.1 ± 7.6 and 47.5 ± 9.2 s^{-1}). We therefore show that k_{tr} can be measured in intact rat and rabbit cardiac trabeculae, and that the k_{tr} decreases when muscles are stretched to their optimal length under near-physiological conditions, indicating that the Frank–Starling mechanism not only increases force but also affects cross-bridge cycling kinetics.

INTRODUCTION

The main function of the heart is to pump blood to meet the demands of the body. This pumping activity depends on cardiac muscle contraction, which, in turn, depends on the interaction of sarcomeric thick and thin filaments, which form cross-bridges that generate force. Consequently, the pumping capability of the heart is determined by the number of cross-bridges capable of generating force and the rate at which they cycle through unbound, weakly bound, and strongly bound (force-generating) states (Hanft et al., 2008; McDonald, 2011). Therefore, alterations in either of these two factors can affect cardiac function.

The Frank–Starling law of the heart—as ventricular volume (corresponding to muscle length) increases, the heart intrinsically strengthens—describes a well-known cardiac regulatory mechanism. Although increased muscle length generally results in improved force development, in parallel with a prolonged time to peak (TTP) force and a slowing of relaxation time (Allen and Kentish, 1985; Monasky et al., 2008, 2010), it is unclear whether changes in muscle length per se affect cross-bridge kinetics. Some previous studies found that cross-bridge cycling kinetics decreased with increased sarcomere length (Adhikari et al., 2004; Stelzer and Moss, 2006;

Korte and McDonald, 2007), whereas others found that sarcomere length has no effect on the rate of cross-bridge cycling (Hancock et al., 1993; Edes et al., 2007). These previous studies, however, were performed using permeabilized cardiac preparations, sub-physiological temperatures, or both. Data obtained under physiological temperature and in intact muscle preparations might help resolve this discrepancy and clarify the effects of muscle length on cross-bridge kinetics.

Various laboratory techniques have been used to study cross-bridge cycling, including Edman's slack test, actomyosin ATPase activity, rate of tension redevelopment, and sinusoidal perturbation (Ruf et al., 1998; Wannenburg et al., 2000; Brixius et al., 2003). Of these, the rate of tension redevelopment (k_{tr}) (Brenner and Eisenberg, 1986) has been the most widely adopted approach. This technique assesses the rate at which force redevelops after a rapid slack–stretch maneuver has disconnected all cross-bridges. The k_{tr} protocol has been used by many investigators and has provided valuable information with regard to quantifying the kinetic steps in thick and thin filament interactions. However, intact

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cardiac preparations do not normally produce tetanic (fused) contractions, even at very high stimulation rates (Slabaugh et al., 2012). This makes measuring k_{tr}, which requires a stable level of Ca^{2+} activation, very difficult. The few studies that measured k_{tr} in preparations with intact membranes (Hancock et al., 1993; Baker et al., 1998; Hannon et al., 2001) combined high frequency stimulation with irreversible SR poisoning (using cyclopiazonic acid or ryanodine) to maintain stable Ca^{2+} concentrations, an approach that is constrained to low (nonphysiological) temperatures. Our goal here was to design a protocol that allows repeated assessment of k_{tr} in intact cardiac trabeculae at physiological body temperature. We found that this could be done reliably and reproducibly by using K^+ contractures, a technique that leads to depolarization of the muscle, causing an influx of calcium into the cytoplasm that produces a tetanus-like steady-state contraction. We then used this method to show that an increase in cardiac muscle length leads to a decrease in k_{tr}.

MATERIALS AND METHODS

Animal model and trabeculae isolation

For the first part of the study, we assessed three muscles from rabbit hearts. Rabbits were anesthetized using 50 mg/kg sodium pentobarbital, delivered intravenously (into the lateral ear vein). However, we used rats for most experiments. Male brown Norway rats ($\sim$3 mo old and weighing 250 g; $n = 11$) were anesthetized intraperitoneally with 50 mg/kg sodium pentobarbital. The chest wall was opened by means of bilateral thoracotomy, and the heart was injected with 1,000 U heparin. In all cases, the heart was rapidly removed and perfused via the ascending aorta with Krebs–Henseleit solution containing (mM) 137 NaCl, 5 KCl, 10 glucose, 20 NaHCO$_3$, 1.2 MgSO$_4$, 1.2 NaH$_2$PO$_4$, 0.25 CaCl$_2$, and 20 2,3-butanedione monoxime (BDM) (Bupha-Intr et al., 2009; Slabaugh et al., 2012). The BDM prevents contractions and minimizes cutting injury during dissection (Mulieri et al., 1989). The Krebs–Henseleit solution was equilibrated with 95% O$_2$/5% CO$_2$ resulting in a pH of 7.4. The right ventricle was opened, and thin

nonbranched trabeculae (average dimensions of 159 ± 11–µm wide, 106 ± 7–µm thick, and 1.5 ± 0.1–mm long; $n = 11$; rat) were dissected leaving free ventricular wall at both ends. Muscles with a thickness of >150 µm were excluded from analysis to avoid the effects of core hypoxia (Raman et al., 2006).

Experimental apparatus

Muscles were mounted in a custom-made bath and connected to a force transducer (Scientific Instruments Heidelberg) on one end by means of two parallel hooks (to eliminate rotation movement artifacts) and to a linear motor (Scientific Instruments Heidelberg) (Xu et al., 2011a,b) on the other end. Vibrations associated with the movement of the motor and the flow of the superfusate were reduced by placing a small glass slide over the bath, and an electronic signaling anti-oscillation unit with an effective time constant faster than 1.2 ms was used to improve signal resolution (Scientific Instruments Heidelberg) (Xu et al., 2011a). The muscles were perfused with Krebs–Henseleit solution as described in the section above (without BDM and containing 2.0 mmol/L CaCl$_2$). The solutions were kept at a constant temperature of 37°C and equilibrated with 95% O$_2$/5% CO$_2$. Rat and rabbit muscles were stimulated at 4 and 1 Hz, respectively. The optimal lengths of the muscles were determined as described previously (Janssen et al., 2002). Clear striation patterns cannot always be observed with intact trabeculae preparations, but previous work (Rodriguez et al., 1992) has shown that optimal length (L$_{opt}$) corresponds to a sarcomere length of $\sim$2.2 µm, which is close to the sarcomere length at the end of diastole.

Experimental protocol

The rate of tension redevelopment was measured for each rat muscle at both the optimal length (L$_{opt}$) and at a shorter length, L$_{90}$ (90% of L$_{opt}$), close to the in vivo sarcomere length at the end of systole. To determine whether experimental order affected the results, we measured k_{tr} in the following order: L$_{opt}$→L$_{90}$→L$_{opt}$→L$_{90}$ in one subset of rat muscles ($n = 6$) and L$_{90}$→L$_{opt}$→L$_{90}$→L$_{opt}$ in a second set ($n = 5$). The K$^+$ contracture plateau (peak) allows for a steady-state equilibrium between calcium and force (Varian et al., 2006). Therefore, we performed all k_{tr} experiments when the muscles were under maximal force–inducing K$^+$ contracture, conditions under which calcium concentration is 1 µM or higher, which is saturating for force in intact preparations (Varian et al., 2006, 2009; Monasky et al., 2010). After the muscles were maintained in Krebs–Henseleit solution for 15–20 min at either L$_{opt}$ or L$_{90}$, we induced K$^+$ contracture by switching to a solution containing
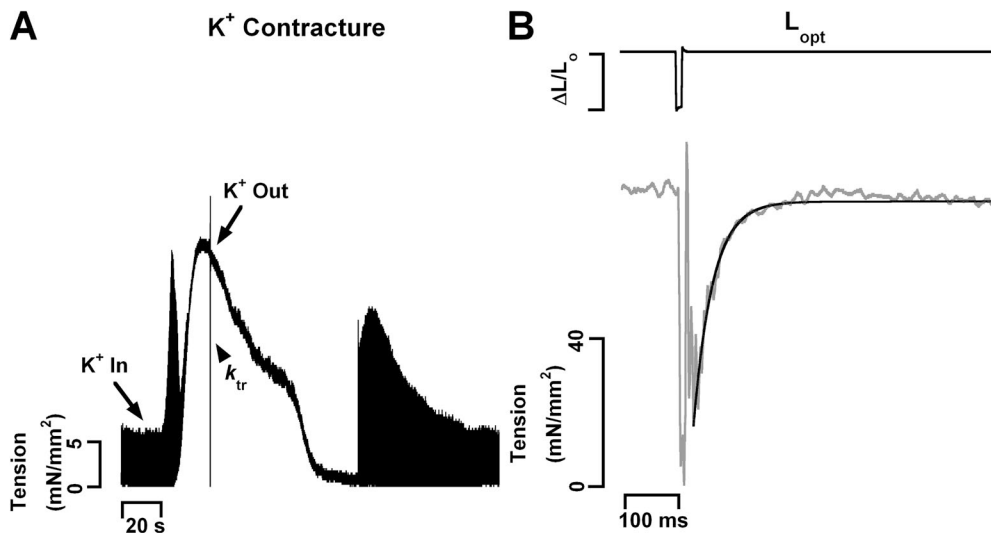
Figure 1. Tracings of K$^+$ contracture and ktr protocols performed in intact rat trabeculae. (A) Representative K$^+$ contracture in an intact rat muscle preparation at L$_{90}$. The k_{tr} protocol was executed at the plateau stage of each contracture (indicated by arrowhead). Changes in K$^+$ concentration are indicated by arrows. (B) Representative k_{tr} tracing in a single intact muscle at L$_{opt}$ (value amounted to 28.2 s^{-1}). The motor position is shown at the top of the tracing.

(mM) 121.4 KCl, 20.6 NaCl, 10 glucose, 20 $NaHCO_3$, 1.2 $MgSO_4$, 1.2 NaH_2PO_4, and 6 $CaCl_2$. Once the K^+ contracture started, the stimulation was turned off and the contracture was allowed to reach its plateau (maximum tension) phase. At this point the k_{tr} was determined, and the solution was rapidly switched back to Krebs–Henseleit buffer (inducing relaxation of the K^+ contracture) and the electrical stimulation was restarted. Muscle length was then adjusted to L_{opt} or L_{90} as appropriate, and the preparation was allowed to stabilize at the new length for 15–20 min, after which the K^+ contracture and determination of k_{tr} protocol were repeated. A typical tracing of an entire K^+ contracture containing a k_{tr} is given in Fig. 1 A for a muscle at L_{90}. An example of a k_{tr} tracing is given in Fig. 1 B. k_{tr} was determined through the following protocol: the muscle was rapidly shortened to 80% of its length during 1 ms ("slack" phase), maintained at this length for 10 ms, and then rapidly restretched to the original length during 1 ms. This maneuver disrupts cross-bridges, resulting in a decrease in force, followed by an exponential increase in force as the cross-bridges reattach (Brenner and Eisenberg, 1986; Campbell et al., 2003). Data were collected at a rate of 10 kHz.

Data analysis and statistics

All data were collected be means of a custom-made application in LabView (National Instruments). The cross-sectional areas of the muscles were used to normalize absolute force measurements to reduce variations among muscles of different diameter. The rate of tension redevelopment (k_{tr}) was obtained by fitting the force redevelopment curve following the slack–restretch maneuver to the equation $F = F_{max} \cdot (1 - e^{-ktr(t)}) + F_{initial}$ (in which F = force, F_{max} = maximal force, and $F_{initial}$ = initial force) using a nonlinear least-squares fitting method (Kemmer and Keller, 2010). Fitting a double exponential did not significantly improve the fit. Additionally, the half-time of force redevelopment, $t_{1/2}$, was determined by linear transformation of the data and used for calculation of k_{tr}, where $k_{tr} = \ln(2) \cdot (t_{1/2})^{-1}$. The differences between multiple groups were analyzed via two-way ANOVA with a significance threshold of P < 0.05. The differences between k_{tr} calculated by monoexponential curve fit and linear transformation were determined by paired Student's t test with a significance threshold of P < 0.05. The data are presented as mean ± SEM.

RESULTS

Intact muscle can be used to assess k_{tr} in rat and rabbit myocardium

First, we compared k_{tr} in two species: the rat, which expresses the fast α-myosin isoform, and the rabbit, which expresses the slow β-myosin isoform. In Fig. 2 (A and B), we show traces of k_{tr} performed in a rabbit muscle. We obtained typical force tracings, similar to those described in permeabilized muscles at sub-physiological temperatures (Brenner and Eisenberg, 1986). Analyses of duplicate measurements showed that results were repeatable and reproducible (Fig. 2 C). k_{tr} is considerably slower (10.6 ± 1.2 s^{-1}; $n = 3$) in rabbit than in rat (27.7 ± 3.3 s^{-1}; $n = 11$; P < 0.01) under identical conditions (Fig. 2 D), indicating that the rate of k_{tr} at physiological temperature differs with different myosin isoforms.

Next, we investigated the effect of temperature on k_{tr}. We observed an increase in k_{tr} as temperature was increased from 27 to 37°C ($n = 4$ trabeculae, each from
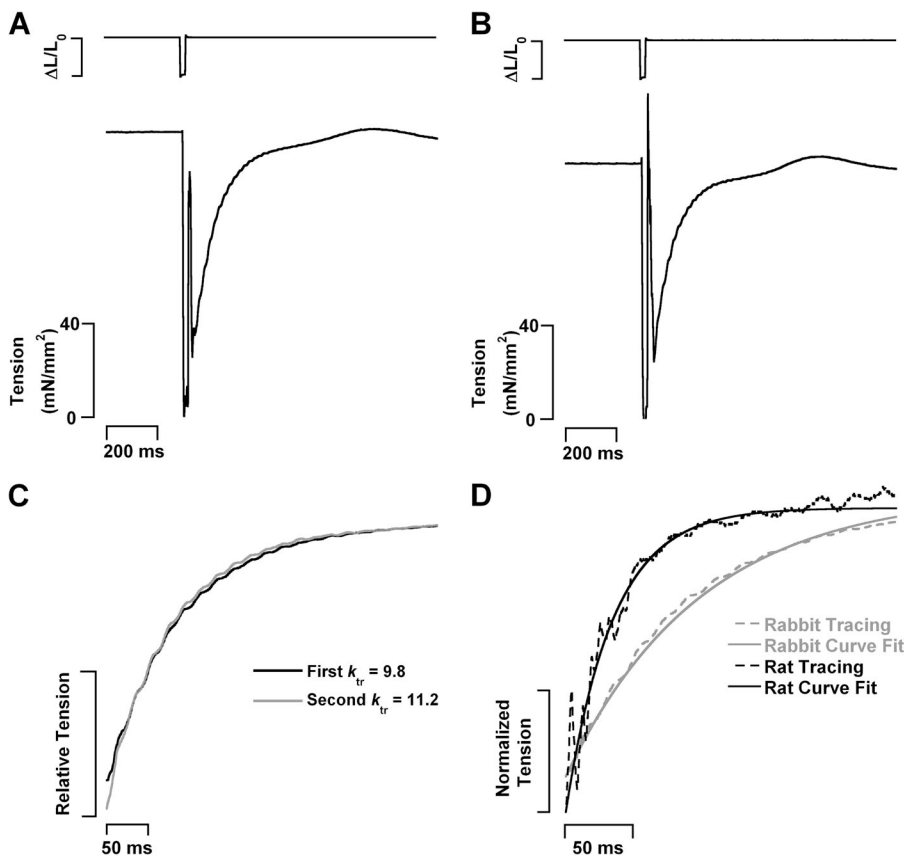

Figure 2. The ktr protocol can be performed in intact rabbit myocardium. (A and B) Consecutive k_{tr} tracings in a rabbit trabecula at L_{opt}. (C) Detail of duplicate k_{tr} tracings shows the repeatability and reproducibility of these analyses. (D) A comparison with rat trabeculae, under otherwise identical conditions, shows that rabbit k_{tr} (∼10/s) is significantly slower than rat (∼30/s) (representative of $n = 11$ rat, $n = 3$ rabbit).

different rats), using a stimulation frequency of 1 Hz. We measured a Q10 in the range similar to that observed in permeabilized preparations (average Q10 of 2.2, ranging from 1.9 to 2.8; not depicted). The temperature dependence of our k_{tr} measurements supports the notion that k_{tr} in our experiments reflects cross-bridge cycling kinetics in a similar way as it does in permeabilized preparations at sub-physiological temperature.

Increase in muscle length increases maximal tension and reduces k_{tr}

Stretching the muscle from L_{90} to L_{opt} resulted, as expected, in a significant increase in twitch tension (Fig. 3 A) from 17.0 ± 2.8 to 30.9 ± 3.3 mN/mm^2 (L_{90} vs. L_{opt}, respectively; P < 0.05). In addition, at longer length, as expected (Janssen, 2010a,b), the TTP (Fig. 3 B), which measures the time it takes for maximal twitch tension to develop, was prolonged from 50.2 ± 1.7 ms at L_{90} to 55.7 ± 2.2 ms at L_{opt} (P < 0.05). Similar results were observed for RT$_{50}$, which is the time from peak twitch force to 50% relaxation (Fig. 3 C), which increased from 30.2 ± 1.6 to 37.5 ± 1.5 ms (P < 0.05). The increase in muscle length resulted in an increase in the maximal tension obtained during the K$^+$ contracture. The maximum (plateau) K$^+$ contracture tension was 32.7 ± 5.1 mN/mm^2 at L_{90} and 67.2 ± 6.6 mN/mm^2 for L_{opt} (Fig. 4 A). Maximum K$^+$ contracture tension was not affected by time-dependent rundown; repeat measurements showed similar values (35.3 ± 8.0 mN/mm^2 for L_{90} and 61.2 ± 7.0 mN/mm^2 for L_{opt}; P = 0.80). Maximum K$^+$ contracture tension between L_{90} and L_{opt} was significantly different (P < 0.05).

K_{tr} decreased as muscles were stretched from L_{90} to L_{opt} (see example in Fig. 4 B). The average rate of tension redevelopment was 45.1 ± 7.6 s^{-1} at L_{90} and 27.7 ± 3.3 s^{-1} at L_{opt} (Fig. 4 C). When k_{tr} for each length was measured a second time, the repeat k_{tr} measurements were 47.5 ± 9.2 s^{-1} for L_{90} and 27.8 ± 3.0 s^{-1} for L_{opt}, indicating a high reproducibility (Fig. 4 C). K_{tr} was significantly different between the two lengths (P < 0.05), but similar between the initial and repeat measurements at each length, P = 0.84. Quantification of the k_{tr} data by linear transformation yielded values in close agreement and not significantly different from the above data (P > 0.4; not depicted). Finally, analysis of residual tension (F$_{res}$) after k_{tr} revealed a ratio of F$_{res}$ to F$_{dev}$ of 0.07 ± 0.05 at L_{90}, and this ratio was not significantly (ANOVA; P = 0.85) different from that at L_{opt} (0.09 ± 0.05).

DISCUSSION

We have developed a method for studying the effect of muscle length on cross-bridge cycling kinetics in intact cardiac trabeculae at physiological temperatures. We found that (a) it is feasible to assess in k_{tr} repeatedly in intact muscle preparations at physiological temperature using K$^+$ contractures; and (b) under these conditions, an increase in rat muscle length leads to a decrease in k_{tr}.

We found effects of both different myosin isoforms and different temperature on cross-bridge kinetics similar to those described previously in permeabilized muscle at sub-physiological temperatures. k_{tr} for the α-myosin isoform was significantly faster than with the β isoform (Bottinelli et al., 1994; Herron et al., 2001), and increased temperature sped up k_{tr} (Hancock et al., 1996; de Tombe and Stienen, 2007). Given a Q10 of $\sim$2–3, the rate of tension redevelopment in our studies (up to 45–50 s^{-1}) would virtually be identical to six previously reported values (average of $\sim$9 s^{-1} and range of 7–13 s^{-1}) for rats in skinned preparations at colder temperatures (Wolff et al., 1995; Hancock et al., 1996; Fitzsimons et al., 2001; Adhikari et al., 2004; Chen and Ogut, 2006; Chandra et al., 2007) and also be very close to those obtained in intact SR-poisoned cardiac trabeculae, where k_{tr} at normal calcium was $\sim$11 s^{-1} (Baker et al., 1998).
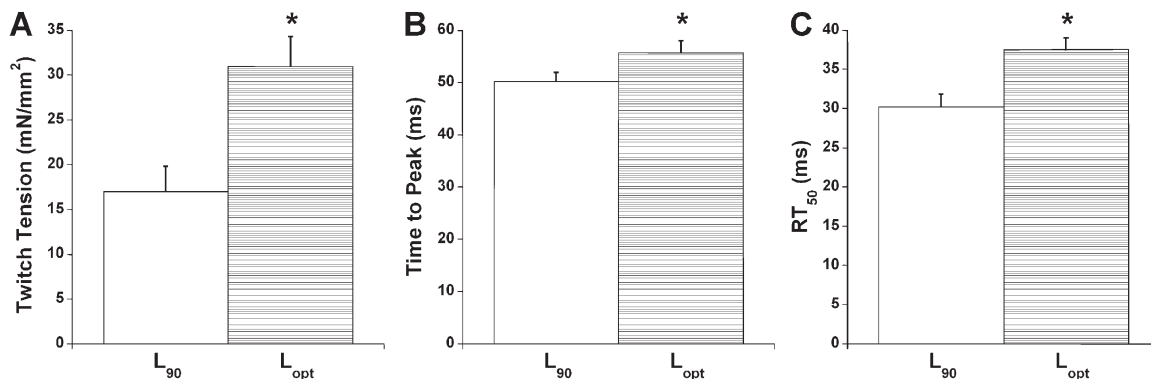

Figure 3. Increasing muscle length results in an increase of twitch force and prolongation of twitch kinetics. (A) Rat muscle twitch tension increases with length (P < 0.05). (B) TTP is greater at L_{opt} than at L_{90} (P < 0.05). (C) RT$_{50}$ (time from TTP to 50% relaxation) increases significantly as muscles are stretched to L_{opt} (P < 0.05). *, differences between L_{opt} and L_{90}. Data are represented as mean ± SEM ($n = 11$).

Permeabilized or "skinned" preparations, which have typically been used to determine cross-bridge kinetics, have produced a wealth of critical knowledge. Although they are ideally suitable for highly controlled experiments on cross-bridge kinetics, these preparations are devoid of posttranslational modification machinery because membranous structures have been (partially) removed. This in turn may render inactive or altogether removes signaling kinases and phosphatases. However, posttranslational modification of contractile proteins is encountered under different conditions of preload, frequency, and β-adrenergic stimulation, and it has been proposed as a mechanism for altering cross-bridge cycling dynamics (Kranias and Solaro, 1982; de Tombe, 2003; Tong et al., 2004; Layland et al., 2005; Lamberts et al., 2007; Varian and Janssen, 2007; Ait Mou et al., 2008; Hidalgo et al., 2009; Varian et al., 2009; Monasky et al., 2010).

We used a modified K^+ contracture protocol (Holubarsch, 1983; Varian et al., 2006; Varian and Janssen, 2007; Monasky et al., 2010) to reversibly "tetanize" intact cardiac trabeculae to assess cross-bridge kinetics at physiological temperature. This type of contracture induces a reversible steady-state force, without the need for compounds that interfere with SR calcium cycling (Hancock et al., 1993; Gao et al., 1994; Baker et al., 1998; Hannon et al., 2001), and can be repeated many times in the same muscle. The maximum tension developed during the K^+ contracture did not change between duplicate measurements; this suggests that the maximal force generating capacity of myofilaments was not affected by a prior measurement per se, nor by the passage of time during the course of our analyses. Furthermore, we found that maximum tension at a given muscle length was independent of the order of length changes.

Although the relationship between muscle or sarcomere length and force development is well known

(Allen and Kentish, 1985), the effect of sarcomere length on cross-bridge cycling rate remains controversial. We found that, when intact muscle length is reduced to 90% of optimal length, the rate of tension redevelopment was significantly accelerated. This is consistent with previous studies that used permeabilized cardiac preparations at sub-physiological temperatures (Adhikari et al., 2004; Stelzer and Moss, 2006; Korte and McDonald, 2007). However, other studies indicated that sarcomere length has no effect on rate of cross-bridge cycling (Hancock et al., 1993; Edes et al., 2007). The different results obtained in these studies could stem from various sources. First, they reflect experiments performed with different animal species or strains. Additionally, all but one (Hancock et al., 1993) of the past studies used permeabilized cardiomyocytes, which do not fully recapitulate intact myocardium. For instance, they do not have constant volume behavior when stretched; the interfilament spacing upon stretch may not reduce as much in skinned preparations compared with a similar stretch in intact muscle. Furthermore, many of these experiments were performed at a temperature range of 12–27°C, at which the behavior of many physiological processes may differ from that at mammalian physiological temperatures ($\sim$37°C) (Little et al., 2012). Moreover, posttranslational modification of myofilament targets influences contractile properties, and the in situ status of posttranslational modifications may be (partially) lost with preparation of the muscle or myocyte for in vitro experimentation. As a result, assessment of cross-bridge cycling rate could thus be affected by preparation-induced or reduced levels of such modifications (Marston and de Tombe, 2008; Monasky et al., 2010). In addition, myofilament compliance may affect cross-bridge cycling rate (Martyn et al., 2002), and the presence of compliant structures, such as collagen and titin, in muscle preparations may render k_{tr} rates at different
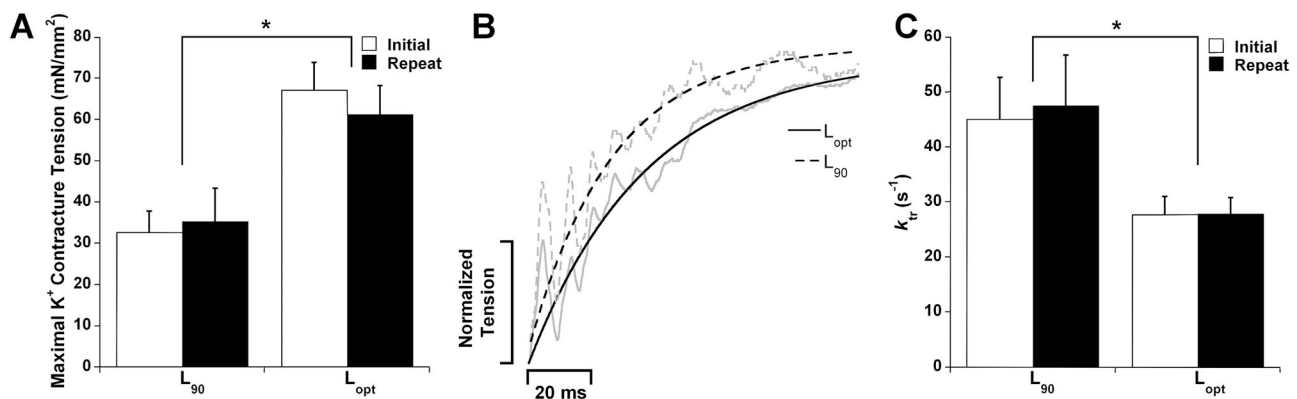

Figure 4. Increase in muscle length decreases ktr in intact rat trabeculae. (A) Increase in muscle length is associated with a significant increase in maximal tension during K^+ contracture (*, $P < 0.05$). (B) Superimposed k_{tr} tracings of L_{opt} and L_{90} in a single rat muscle. The tracings show the initial 100 ms of force redevelopment. (C) Increasing muscle length results in a decrease in k_{tr} (*, $P < 0.05$). The tensions and k_{tr} were not significantly different between duplicate measurements of each group ($P = 0.80$ [tension] and $P = 0.84$ [k_{tr}]). Data are represented as mean ± SEM ($n = 11$).

levels of force apparent rather than absolute. Finally, the residual tension could have an effect on the k_{tr} (Campbell, 2006; Campbell and Holbrook, 2007); however, residual tension was not significantly different in our studies and therefore could not have contributed to the lower k_{tr} observed at longer muscle lengths. At present, we do not have the necessary information and data to propose a molecular mechanism for the acceleration of k_{tr} we observed at shorter muscle lengths. However, others (Korte and McDonald, 2007) have proposed plausible mechanisms to explain this phenomenon. For instance, at longer muscle length, titin induces a strain on myosin-binding protein C, which in turn restrains the movement of myosin heavy chains, thus decreasing cross-bridge cycling rate (Korte and McDonald, 2007).

In conclusion, our work reveals that repeated k_{tr} measurements are feasible in intact myocardium at body temperature and that in intact muscle, k_{tr} decreases with increasing muscle length.

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