Effects of Rapamycin on Cardiac and Skeletal Muscle
Contraction and Crossbridge Cycling

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Rapamycin and myofilament contraction

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Abbreviations:
mTOR, mammalian target of rapamycin; FK506, tacrolimus; F-actin, filamentous actin; FHC, familial hypertrophic cardiomyopathy; PI3K, phosphoinositide 3-kinase; dnPI3K, dominant negative PI3K; FKBP12, FK506 binding protein; S6K1, p70 ribosomal S6 kinase 1; RyR, ryanodine receptor; HMM, heavy meromyosin; RhPh, rhodamine phalloidin; NFAT, nuclear factor of activated T-cells

Recommended section assignment: Cardiovascular
ABSTRACT

The immunosuppressant drug rapamycin attenuates the effects of many cardiac hypertrophy stimuli both in vitro and in vivo. While rapamycin’s inhibition of mTOR and its associated signaling pathways is well established, it is likely that other signaling pathways are more important for some forms of cardiac hypertrophy. Considering the central role of myofilament protein mutations in familial hypertrophic cardiomyopathies, we tested the hypothesis that rapamycin’s anti-hypertrophy action in the heart is due to direct effects of the drug on myofilament protein function. We found little or no effect of rapamycin (10⁻⁸ – 10⁻⁴ M) on maximum Ca²⁺-activated isometric force, while Ca²⁺-sensitivity was increased at some rapamycin concentrations in rabbit skeletal and cardiac, and rat cardiac muscle. At concentrations that increased Ca²⁺-sensitivity of isometric force, rapamycin reversibly inhibited kinetics of isometric tension redevelopment (k_TR) in rabbit skeletal, but not cardiac, muscle. The greatest inhibition (~50%) was at intermediate levels of Ca²⁺-activation, with less inhibition of k_TR (~15%) at maximum Ca²⁺ activation levels. Rapamycin (10⁻⁷ M) increased F-actin sliding speed (~11%) in motility assays, but inhibited sliding at 10⁻⁵ – 10⁻⁴ M. These results indicate that rapamycin has a greater effect on Ca²⁺ regulatory proteins of the thin filament than on actomyosin interactions. These effects, however, are not consistent with rapamycin’s anti-hypertrophic activity being mediated through direct effects on myofilament contractility.
INTRODUCTION

Rapamycin (sirolimus) is an antibiotic derived from *Streptomyces hygroscopicus* that is most commonly used as an immunosuppressant in the treatment of human organ transplant patients. Rapamycin’s antigrowth properties may also be clinically useful for cardiovascular problems. Stents impregnated with rapamycin effectively reduce restenosis (Woods and Marks, 2004). Rapamycin may also prove useful in treating atherosclerosis (Castro et al., 2004).

Furthermore, *in vitro* studies with cardiac myocytes show that rapamycin inhibits both protein synthesis and cardiac myocyte hypertrophy (Sadoshima and Izumo, 1995; Boluyt et al., 1997). At clinically relevant doses, rapamycin attenuates development of cardiac hypertrophy and regresses established hypertrophy in aortic-banded mice without reducing cardiac function (Shioi et al., 2003; McMullen et al., 2004). The increase in cardiomyocyte size typically seen in aortic-banded mice is also effectively attenuated by rapamycin. The relevant biochemical pathway(s) affected by rapamycin have not been unambiguously identified; there are several possibilities.

Rapamycin is an analog of the anti-rejection drug FK506 (tacrolimus) and can form a complex with FK506-binding protein (FKBP12) (Marks, 1996). The rapamycin/FKBP12 complex is an inhibitor of the mammalian target of rapamycin (mTOR), a key downstream component of the PI3K pathway (Frey and Olson, 2003). mTOR is a 290 kDa Ser/Thr kinase that controls mammalian protein translational processes that are central to cell growth (Thomas and Hall, 1997; Schmelzle and Hall, 2000). mTOR exerts its translational control partially through activation of p70 ribosomal S6 kinase, or S6K1 (Schmelzle and Hall, 2000). Rapamycin has been shown to be a strong inhibitor of S6K1 activation, presumably through its inhibition of mTOR (Chou and Blenis, 1995). There are downstream effectors and targets of mTOR in addition to S6K1 (e.g., 4EBP1), although there is little *in vivo* data from mouse heart beyond
changes in S6K1 phosphorylation and activity. Rapamycin’s anti-hypertrophic activity in aortic-banded mice is consistent with inhibition of the PI3K pathway (mTOR) causing suppression of load-induced S6K1 activation (Shioi et al., 2003). Studies with dnPI3K transgenic mice, however, indicate that the PI3K pathway does not play a role in pathological cardiac hypertrophy induced by aortic banding (McMullen et al., 2003). While it remains possible that mTOR activation in these studies occurs through PI3K-independent pathways, alternative routes for rapamycin’s anti-hypertrophic activity should be considered.

Binding of rapamycin to FKBP12 also alters Ca\(^{2+}\) transients in myocytes by dissociating FKBP12 from ryanodine receptors (RyR). Interestingly, this alteration differs between rabbit and mouse ventricular myocytes; rapamycin increases the Ca\(^{2+}\) transient in mouse, but decreases it in rabbit myocytes (Su et al., 2003). Unlike FK506, rapamycin is not a direct inhibitor of calcineurin. Calcineurin is a Ca\(^{2+}\)-calmodulin dependent Ser/Thr phosphatase which alters NFAT-dependent gene transcription and has been shown to participate in aortic-banding induced cardiac hypertrophy (Frey and Olson, 2003; Wilkins et al., 2004). Thus, rapamycin could influence the calcineurin-dependent hypertrophic response by altering Ca\(^{2+}\) release, and therefore intracellular Ca\(^{2+}\). There is, however, at least one additional possibility that may plausibly explain rapamycin’s anti-hypertrophic effects in the heart.

Inherited forms of hypertrophic cardiomyopathies have been linked to mutations in several cardiac myofilament proteins including myosin heavy and light chains, and the Ca\(^{2+}\) regulatory proteins tropomyosin, troponin I and troponin T (Bonne et al., 1998; Fatkin and Graham, 2002). The signaling pathways by which these mutations trigger the hypertrophic response are unknown. The existence of these disease-causing mutations raises the possibility that rapamycin’s anti-hypertrophic effects in cardiac tissue could be due, in part, to a direct effect
of rapamycin on the myofilaments and myocyte contractility. Many FHC-related mutations enhance contractility by increasing Ca$^{2+}$-sensitivity of isometric force and/or unloaded sliding speed, and in some instances by also increasing maximum sliding speed (Lin et al., 1996; Sweeney et al., 1998; Homsher et al., 2000; Takahashi-Yanaga et al., 2001; Fatkin and Graham, 2002; Köhler et al., 2003). Therefore, if rapamycin’s anti-hypertrophic activity was mediated in part by direct effects on myofilament proteins, we predicted that rapamycin should have effects opposite to those of FHC mutations: rapamycin would reduce force and/or sliding speed at a given [Ca$^{2+}$].

In the present study, we explored the acute effects of rapamycin on isometric force and Ca$^{2+}$-sensitivity in skinned skeletal and cardiac muscle, as well as its effects on F-actin sliding speed in the in vitro motility assay. Our data indicate that rapamycin has little or no effect on maximum Ca$^{2+}$-activated force, and inhibits F-actin sliding speed only at high concentrations. At concentrations where rapamycin affected submaximal force, it increased Ca$^{2+}$-sensitivity. These results suggest that rapamycin’s anti-hypertrophic effects do not involve direct inhibition of the myofilament proteins.
MATERIALS and METHODS

Animal Use. Animal handling was in accordance with the current NIH/NRC Guide for the Care and Use of Laboratory Animals. All procedures and protocols were approved by Florida State University’s Institutional Animal Care and Use Committee (IACUC).

Fiber Mechanics

Preparation of single, glycerinated fibers. Single skeletal fiber segments from male New Zealand white rabbit psoas muscles were dissected and prepared for mechanical experiments as described previously (Chase and Kushmerick, 1988; Chase et al., 1994; Regnier et al., 2002). The bundles were permeabilized with 0.5% Brij 58, then 50% glycerol in skinning solution and were stored at -20°C before use (Regnier et al., 2002). Trabeculae from male Sprague-Dawley rat hearts and male New Zealand white rabbit hearts were dissected and prepared as described previously for rat cardiac trabeculae (Regnier et al., 2000). Hearts were excised, and the interior wall of the right ventricle was exposed to relaxing solution containing glycerol (50% v/v) and Triton X-100 (1%) overnight at 4°C. (Unless otherwise indicated, all chemicals used in experiments were supplied by Sigma-Aldrich Co. St. Louis, MO).

Permeabilized cardiac trabeculae were then dissected and stored at 4°C for up to 4 days.

Skeletal fiber ends were chemically fixed by applying glutaraldehyde (5% in H$_2$O with 1 mg/ml Na fluorescein added as a visual indicator) from a syringe flow system. The fixed ends were wrapped in aluminum foil T-clips for attachment to the mechanical apparatus via small wire hooks. Fiber segments were mounted on a motor (step time 300 µs) (Aurora Scientific Inc, Ontario, Canada) to control overall fiber length at one end, and on a capacitative-type force transducer ($f_c = 3.3$ kHz; Aurora Scientific Inc, Ontario, Canada) at the other end. The motor and
force transducer were located on a modified stage of an inverted microscope (Chase and Kushmerick, 1988).

Initial sarcomere length (Ls) (measured by helium-neon laser diffraction), fiber length and fiber diameter (measured with an optical micrometer) were measured prior to the start of each experiment. Trabecular Ls was set to 2.2 µm at pCa 9.0; skeletal fiber Ls was set to 2.6 µm. Chemical fixation of fiber segment ends and reduced temperature were utilized to maintain integrity of fiber structure and mechanical properties during prolonged activation. Both experimental control systems also employed periodic (0.2 Hz) unloading (i.e., fiber shortening followed by restretch to the isometric length) (Brenner, 1983).

**Solutions.** Relaxing and activating solutions were prepared as described previously (Chase and Kushmerick, 1988; Martyn and Gordon, 1988) and contained 5 mM MgATP, 15 mM phosphocreatine (PCr), 1mM Pi, 10 mM EGTA, 50 mM MOPS, 45 mM free Na+, 100 mM free K+, 1 mM DTT, 200-400 units/ml creatine phosphokinase (CK), and 4% w/v Dextran T-500 (Fisher, Pittsburgh, PA). Ca²⁺ solutions were prepared at evenly spaced pCa’s (pCa = -log [Ca²⁺], where [Ca²⁺] is in molar) ranging from pCa 9 (relaxing) to pCa 5 and also pCa 4.5 (maximally activating). To alter solution [Ca²⁺], appropriate amounts of Ca(acetate)₂ were added, taking into account desired free [Ca²⁺] and the binding constants of all solutions constituents for Ca²⁺. Ionic strength was maintained constant (180 mM) by varying Tris and acetate. Solution pH was adjusted to 7.0 at 12°C. Rapamycin (LC Laboratories, Woburn, Ma, R-5000) was reconstituted in 100% absolute ethanol and added to the varying pCa solutions for a final concentration of 1% ethanol and specified rapamycin concentration. Final concentrations of rapamycin ranged from 10⁻⁸ M to 10⁻⁴ M. 1% ethanol was present in all solutions to control for possible effects of ethanol alone (Regnier et al., 1996).
Experimental solutions were held in 200-µl anodized aluminum wells. The bottom of each well consisted of a glass number 1 cover slip. The temperature was set at 10°C for skeletal fibers and 15°C for cardiac fibers, and was controlled to within 1°C during individual experiments using an ATR-4 adaptable thermoregulator (Quest Scientific, North Vancouver, BC Canada).

**Experimental control, data acquisition, and data analysis.** Steady-state isometric force and the kinetics of force redevelopment were determined as described previously (Chase et al., 1994; Regnier et al., 2002) (Fig. 1). Data were discarded if maximum Ca\(^{2+}\)-activated force in control conditions (no rapamycin) decreased more than 20% of the initial control. Force redevelopment kinetics \(k_{TR}\) were characterized by obtaining an apparent rate constant (expressed as reciprocal seconds) from the linear transformation of the half-time estimate \(t_{1/2}\) extrapolating from 50% to 63.2% \(k_{TR} = \tau^{-1} = -\ln 0.5 \cdot (t_{1/2})^{-1}\) (Regnier et al., 1998). Mono-exponential fits to the data, obtained with the Simplex method for nonlinear least-squares regression, gave similar results.

The relation between steady-state, isometric force (F) and pCa was fitted by a nonlinear least-squares regression to the Hill equation:

\[
\frac{F}{F_0} = \frac{1}{(1 + 10^{n(pCa_pCa_{50})})} \quad \text{Eq. 1}
\]

where \(F_0\) is the maximum force at pCa 5, pCa\(_{50}\) is the pCa at which F = F\(_0\)/2, and \(n\) determines the steepness of the Ca\(^{2+}\) dependence around pCa\(_{50}\).

**In vitro Motility Assays**

**Solutions and Flow Cell Preparation.** *In vitro* motility assays were performed to measure the sliding speed of fluorescently labeled F-actin over heavy meromyosin (HMM) coated surfaces.
Experimental protocols, solution preparation, flow cell preparation, and analysis were essentially as described previously (Chase et al., 2000). Assays were carried out at 30°C, with temperature in the flow cell maintained by circulating temperature-controlled water through a copper coil around the objective. Actin and myosin were extracted from New Zealand White Rabbit back and leg muscles. F-actin was fluorescently labeled with rhodamine phalloidin (RhPh). HMM was obtained by mild chymotryptic digestion of myosin. Immediately before the motility assay, 2 mM ATP, 16.7 mM glucose, 100 µg/ml glucose oxidase, 18 µg/ml catalase (Worthington Biochemical Corp., Lakewood, NJ), 0.3% methyl cellulose, and an additional 40 mM DTT were added to actin buffer (AB), composed of 25 mM KCl, 25 mM imidazole, 4 mM MgCl₂, 1 mM EGTA, 1 mM DTT, at pH 7.4 (Kron et al., 1991). Glucose, glucose oxidase, catalase and extra DTT were added to minimize photo-bleaching of the RhPh label and photo-oxidative damage to the proteins (Kron et al., 1991; Gordon et al., 1997). Rapamycin was reconstituted in 100% absolute ethanol, and added to solutions at concentrations of $10^{-8} – 10^{-4}$ M, with a final concentration of 1% ethanol in all solutions as described for permeabilized muscle preparations. The negative control AB (no rapamycin added) was prepared with 1% ethanol to control for possible effects of ethanol alone (Regnier et al., 1996).

**Fluorescence Microscopy and Data Acquisition.** RhPh labeled F-actin motility was observed by fluorescence microscopy on a Diastar-Reichert microscope at 100X magnification, imaging 6-8 fields from varying areas on the flow cell. Field images were recorded as 30-second clips using a VE1000 SIT camera (Dage-MTI, Inc, Michigan City, IN) and a Panasonic AG-7350 videocassette recorder. The analog clips were digitized using iMovie software as QuickTime files on a Macintosh 2X PowerPC G4 computer using a Miglia video interface (Miglia Technology Ltd., Enfield, UK). RhPh F-actin sliding speed distributions were analyzed.
by custom motion analysis software (designed by Thomas Asbury and P. Bryant Chase, Florida State University). Briefly, individual filament speed was measured by tracking the centroid of filaments. A mean sliding speed for all filament paths ($V_f$) was calculated for each flow cell following removal of paths that had a ratio of standard deviation to mean path speed of $> 0.3$; this procedure effectively omits data from immobile filaments and filaments with erratic movement (Sellers et al., 1993; Gordon et al., 1997; Chase et al., 2000).

**Statistical Analyses.** Statistical analyses, including linear and nonlinear least-squares regression, were performed using Excel (version 2000; Microsoft, Redmond, WA) or SigmaPlot 2002 for Windows (version 8.0; SPSS, Inc, Port Richmond, CA). Student’s t-test was used to compare means.
RESULTS

Maximum Ca\textsuperscript{2+}-activated force. To determine whether rapamycin affects actomyosin interactions, we first measured maximum Ca\textsuperscript{2+}-activated (pCa 5) steady-state isometric force (F\textsubscript{0}) in skinned muscle preparations dipped in bathing solutions with varying rapamycin concentrations. Comparisons were made between rat cardiac and rabbit cardiac muscle, and rabbit fast skeletal muscle fibers. The fibers were dipped in pCa 5 bathing solution (no rapamycin) immediately prior to and following each dip in rapamycin solution to identify irreversible effects of rapamycin and normalize against fiber degradation. There was little or no effect of rapamycin (10\textsuperscript{-8} to 10\textsuperscript{-4} M) on maximum Ca\textsuperscript{2+}-activated force in any of the muscle types (Fig. 2). Given that the inhibitory effects of rapamycin were, at most, small, we chose two concentrations for studies at submaximal Ca\textsuperscript{2+} activation: 1 \textmu M, an effective immunosuppressant therapeutic dosage for mice which is comparable to human dosage (Shioi et al., 2003), and 100 \textmu M, a maximum rapamycin concentration.

Submaximal Ca\textsuperscript{2+} activation of isometric force and kinetics of tension redevelopment. To determine the effects of rapamycin on Ca\textsuperscript{2+}-activation of isometric force, permeabilized preparations were subjected to three consecutive cycles of increasing Ca\textsuperscript{2+} concentrations ranging from pCa 9 to pCa 4.5. The initial control was performed in the absence of rapamycin. Next was a series of measurements with either 1 \textmu M or 100 \textmu M rapamycin. Finally, a repeat of the control measurements was performed to evaluate reversibility of rapamycin effects and to control for degradation of preparations.

We found that rapamycin increased Ca\textsuperscript{2+}-sensitivity of isometric force (leftward shift in the force-pCa relationship, Eq. 1) in some of the experimental conditions studied. In rat cardiac muscle, 100 \textmu M rapamycin increased Ca\textsuperscript{2+}-sensitivity by 0.11 pCa units (Fig. 3C; Table 1),
whereas there was no effect at 1 μM (data not shown). In rabbit cardiac and skeletal muscle, 1 μM rapamycin increased Ca\textsuperscript{2+}-sensitivity by 0.08 or 0.13 pCa units respectively (Fig. 3A, B; Table 1); interestingly, there was no effect at 100 μM in either rabbit muscle type (data not shown).

The kinetics of isometric tension redevelopment were also studied to gain mechanistic insight into the effects of rapamycin at submaximum Ca\textsuperscript{2+} activation (Fig. 1B). $k_{TR}$ data were obtained from rabbit skeletal and rabbit cardiac muscle preparations. To obtain averages of force and $k_{TR}$, data were divided into bins according to the normalized isometric force production (Regnier et al., 1996). Both force and $k_{TR}$ data were normalized to the respective saturating Ca\textsuperscript{2+} value for the same fiber. The maximum $k_{TR}$ was 9.80 ± 0.47 s\textsuperscript{-1} for skeletal muscle and was 1.67 ± 0.13 s\textsuperscript{-1} for rabbit cardiac muscle. $k_{TR}$ increased nonlinearly as force was increased by elevated Ca\textsuperscript{2+}, although the increase was substantially greater in skeletal muscle than in cardiac (Fig. 4). In skeletal muscle, 1 μM rapamycin reversibly inhibited $k_{TR}$ at intermediate force levels under conditions where Ca\textsuperscript{2+}-sensitivity of isometric force was increased (Fig. 4A). At saturating Ca\textsuperscript{2+} levels, $k_{TR}$ was inhibited by ~15%, while the maximum inhibition of $k_{TR}$ observed at intermediate force levels was ~50%. In rabbit cardiac muscle, rapamycin had little or no effect on the relationship between $k_{TR}$ and steady-state force (Fig. 4B).

**Unloaded filament sliding speed.** We utilized an in vitro motility assay with rabbit skeletal actin and HMM to determine if rapamycin affects unloaded sliding speed of F-actin in the absence of calcium regulatory proteins. This method utilizes purified rabbit skeletal F-actin and HMM, and minimizes the potential effects of internal loads that are present in the intact sarcomere studied with fiber mechanics methods. Over the rapamycin concentration range tested, there was a biphasic effect on the F-actin sliding speed (Fig. 5). At 10\textsuperscript{-7} M rapamycin,
V̇ increased by ~11% over the control (no rapamycin). At concentrations of 10^{-5} M or greater, there was a clear inhibition (60-80%) of V̇ (Fig 5).
The purpose of the present investigation was to determine if the beneficial effects of the anti-growth drug rapamycin in animal models of cardiac hypertrophy could be due to direct effects of rapamycin on contractile myofilament function. This possibility derives from the identification of myofilament protein mutations that alter contractility and are causal in cardiac hypertrophy. There were two major findings of this study. First, there was little or no effect of rapamycin on actomyosin crossbridge cycling, except for inhibition in unloaded conditions at concentrations above therapeutic levels. Second, Ca\(^{2+}\) regulation can be altered by rapamycin at therapeutic doses in rabbit tissue, with increased Ca\(^{2+}\)-sensitivity. Thus, the acute effects of rapamycin on myofilament function are inconsistent with the prediction that the drug’s anti-hypertrophic effects stem from inhibition of cardiac contractility.

Our conclusion that rapamycin either has little effect, or enhances cardiac contractility is in agreement with the in vivo data presented by Shioi et al. (2003) and McMullen et al. (2004). Cardiac function of aortic-banded mice was assessed by echocardiography in both studies following administration of rapamycin for 1 week. In mice that were treated with a therapeutic dose of rapamycin prior to and immediately following aortic banding, fractional shortening, and thus the cardiac contractility, was essentially the same in rapamycin-treated and untreated animals (Shioi et al., 2003). In animals where treatment began one week following aortic-banding induced hypertrophy, rapamycin had little or no effect on hearts from animals exhibiting compensated hypertrophy, while it significantly improved fractional shortening and ejection fraction of hearts from animals exhibiting decompensated hypertrophy (McMullen et al., 2004).

**Actomyosin.** Actomyosin interactions were investigated either at maximum Ca\(^{2+}\), when thin filament regulatory proteins troponin and tropomyosin were present, or in the absence of
regulatory proteins. Our data showed little or no effect of rapamycin on maximum Ca\(^{2+}\)-activated force (Fig. 2) or on maximum \(k_{TR}\) (Fig. 4, where force \(\sim 100\%\)). These results indicate that actomyosin crossbridge cycling is not affected by rapamycin under isometric conditions in either steady-state (Fig. 2) or during the approach to steady-state (Fig. 4). The in \textit{vitro} motility assay was utilized to determine if rapamycin affects crossbridge cycling under unloaded conditions. The small but significant enhancement of \(V_f\) at 10\(^{-7}\) M rapamycin and substantial inhibition at \(\geq 10^{-5}\) M (Fig. 5) indicate that rapamycin has a greater effect on unloaded actomyosin crossbridge cycling.

There were no apparent species or muscle type differences in the effects of rapamycin on actomyosin cycling. There was no effect on isometric force measured in all three muscle types examined (Fig. 2). In either the absence or presence of 1 \(\mu\)M rapamycin, rabbit skeletal \(k_{TR}\) was almost 6-fold faster than rabbit cardiac at saturating Ca\(^{2+}\); this kinetic difference is expected because these muscle types contain distinct myosin heavy chain isoforms (Bottinelli and Reggiani, 2000).

\textbf{Ca\(^{2+}\) regulation.} The possibility that rapamycin interacts with calcium regulatory proteins of the thin filament was investigated by determining steady-state force-pCa relationships (Fig. 3 and Table 1) and the Ca\(^{2+}\)-dependence of \(k_{TR}\) (Fig. 4) in the presence and absence of rapamycin. This is particularly relevant because cardiac muscle functions at submaximum Ca\(^{2+}\) levels. Thus rapamycin could significantly influence cardiac physiology if it modulates function of the Ca\(^{2+}\) regulatory proteins, even though there was little or no effect at saturating Ca\(^{2+}\). Rabbit psoas and cardiac preparations showed an increase in Ca\(^{2+}\)-sensitivity of isometric force at 1 \(\mu\)M rapamycin (Fig. 3 and Table 1). There was no effect on the force-pCa relation in either rabbit preparation at a higher dose (100 \(\mu\)M) (data not shown), which may be related to the
biphasic effect on actomyosin cycling observed under unloaded conditions with rabbit skeletal HMM (Fig. 5). A leftward shift was also seen in rat cardiac trabeculae, but only at the higher rapamycin concentration (100 µM) (Fig. 3 and Table 1). These steady-state force data suggest an influence of rapamycin on the thin filament Ca\(^{2+}\)-regulatory proteins.

This conclusion is substantiated by kinetic data (Fig. 4). The greatest effect of rapamycin on \(k_{TR}\) was found at intermediate force levels obtained at submaximum Ca\(^{2+}\) in rabbit psoas fibers (Fig. 4a). \(k_{TR}\) measured at submaximum Ca\(^{2+}\) is an indicator of the dynamics of thin filament regulatory function (Regnier et al., 1996; Regnier et al., 1998) and thus the data in Fig. 4A indicate that rapamycin influences the Ca\(^{2+}\)-regulatory proteins. The \(k_{TR}\)-force relationship in cardiac muscle (Fig. 4b) is not as curvilinear as that for skeletal (Fig. 4a). This difference between cardiac and skeletal muscle has been reported previously and may be due to differences in kinetics of Ca\(^{2+}\) dissociation from TnC, or to differences in coupling between Ca\(^{2+}\) dissociation from TnC and strong crossbridge dissociation from actin (Landesberg and Sideman, 1994; Hancock et al., 1996; Regnier et al., 1996; Regnier et al., 1998). Rapamycin’s inhibition of the \(k_{TR}\)-force relation in cardiac muscle was less than that in skeletal muscle (Fig. 4), although this may be due, at least in part, to regulatory protein isoform differences that underlie cardiac-skeletal differences in the activation-dependence of \(k_{TR}\).

**Mechanism of rapamycin’s anti-hypertrophic activity.** Rapamycin has the ability to suppress load-induced cardiac hypertrophy in aortic-banded mice (Shioi et al., 2003) and to regress established hypertrophy in the same animal model (McMullen et al., 2004). These effects were pronounced, with attenuation of both the increase in total heart weight and increase in myocyte cell size. Many signaling pathways affect cardiac function, and therefore contribute to the causes of cardiac hypertrophy (Fatkin and Graham, 2002; Frey and Olson, 2003; Pyle and
Solaro, 2004). Our data show that rapamycin’s anti-hypertrophic activity cannot be explained by its acute effects on cardiac (and skeletal) muscle mechanics (Fig. 6). This suggests that pathways involving mechano-sensitive signaling molecules, such as those bound to or associated with the Z-disc (red, Fig. 6) (Pyle and Solaro, 2004), are not affected by rapamycin. mTOR, a downstream element of the PI3K pathway (blue, Fig. 6), is important for cell growth. McMullen et al. have shown that although dnPI3K transgenic mice undergo cardiac hypertrophy following aortic banding, they do not exhibit exercise-induced hypertrophy (McMullen et al., 2003). Rapamycin’s ability to attenuate cardiac hypertrophy in aortic-banded mice, therefore, is not likely through the inhibition of PI3K-dependent activation of mTOR.

Ca^{2+} signaling pathways (green, Fig. 6) under control of calcineurin have also been shown to be involved in cardiac hypertrophy (Frey and Olson, 2003). Rapamycin has been shown to dissociate FKBP12 from the ryanodine receptor, which alters Ca^{2+} transients (Su et al., 2003). This alteration could also play a part in the anti-hypertrophic effects seen with rapamycin treatment, and is consistent with the observed upregulation of calcineurin/NFAT gene expression in aortic-banded mice (Wilkins et al., 2004). These same arguments might apply to hypertrophy observed in FHC patients. Many FHC mutations occur in the calcium regulatory proteins of the thin filament, and the majority of them cause an increase in Ca^{2+}-sensitivity in some aspect of cardiac function (Köhler et al., 2003; Wang et al., 2004). If increased Ca^{2+}-sensitivity is associated with increased affinity of Ca^{2+} binding to TnC, then these mutations could also alter Ca^{2+} transients, which would then alter the Ca^{2+}-dependent signaling pathways. Future study of these and other signaling pathways involved in cardiac function should lead to greater understanding of the causes of cardiac hypertrophy, as well as possibilities for treatment and prevention with rapamycin and related compounds.
ACKNOWLEDGMENTS

We thank Lori McFadden and Victor Miller for preparation of myosin, HMM and actin, Dr. Kathleen Harper for assistance with animal anesthesia, and Dr. Fang Wang for assistance with initial motility studies.
REFERENCES


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FOOTNOTES

This work was supported in part by NIH HL63974 and NSBRI MA00211.

Portions of these results have been presented in abstract form (Kataoka et al., 2004).

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FIGURE LEGENDS

Fig. 1. Representative data from an individual, chemically permeabilized rabbit psoas fiber. (A) Chart recording of isometric force generated at pCa 5 in presence (center record) and absence of 100 µM rapamycin (left and right records). The force transients apparent throughout the steady contractures resulted from periodic (0.2 s⁻¹) releases of fiber length (Materials and Methods). (B) Fast time-based digital recordings of force from same fiber shown in panel A. Three force records are superimposed within the graph.

Fig. 2. Effect of rapamycin on maximum Ca²⁺-activated isometric force (pCa 5) in chemically permeabilized preparations from rabbit psoas, and cardiac trabeculae from rabbit and rat. Force in the presence of rapamycin was normalized to the average of that measured before and after. There was little or no effect of increasing rapamycin concentration on force with all three muscle types.

Fig. 3. Rapamycin increases Ca²⁺-sensitivity of steady-state isometric force in (A) rabbit psoas fibers (1 µM rapamycin; N=6); (B) rabbit cardiac trabeculae (1 µM rapamycin; N=6); and (C) rat cardiac trabeculae (100 µM rapamycin; N=8). Each point represents the average force, normalized to that obtained at pCa 5 in the same muscle preparation. Error bars represent the standard error. The curves were generated from the Hill equation (Eq. 1), using average values of pCa₅₀ and n (Table 1) obtained by nonlinear least squares fits to the data from each fiber.

Fig. 4. The effects of rapamycin on activation-dependence of k_TR. Each point represents the averaged k_TR for all muscle preparations analyzed at the given force. Error bars represent
standard error. (A) Rabbit psoas fibers (N=6) showed a marked inhibition of $k_{TR}$ at intermediate force levels. (B) Rabbit cardiac trabeculae (N=8) showed little or no effect on $k_{TR}$ at all force levels.

Fig. 5. Effects of rapamycin on unloaded sliding speed of RhPh-labeled F-actin in the in vitro motility assay. Speed is the average of two flow cells per rapamycin concentration with an average of 160 filaments per flow cell. Error bars represent standard errors of the mean. Dashed line represents the averaged control speed with no rapamycin added to the flow cell. Significance is expressed as * $p < 0.05$ and ** $p < 0.01$, when compared to the averaged control speed. Motility assays were conducted with F-actin and HMM from rabbit skeletal muscle.

Fig. 6. Possible pathways involved in the anti-hypertrophic effects of rapamycin. Rapamycin (yellow) is a known inhibitor of mTOR, a key element of the PI3K pathway (blue), and a putative sensor of cellular energetic status (Dennis et al., 2001). Rapamycin also influences Ca$^{2+}$-dependent pathways (green) through its effects on Ca$^{2+}$ release. In both of these pathways, rapamycin’s action is through binding to FKBP12 (not shown). In this paper, we show that rapamycin’s anti-hypertrophic activity is not through direct effects (“X”) on the myofilament proteins (black) and changes in mechanical activity that could influence signaling molecules that transiently bind to the Z-disc (red). Abbreviations: MLP, muscle LIM protein; Akt, protein kinase B; CaN, calcineurin. Note that this simplified schematic does not attempt to comprehensively illustrate all possible signaling pathways, or additional coupling between pathways. The significant influences between all three subdivisions within the
MYOFILAMENTS heading (energetics, activation, and mechanics) have been omitted for clarity.
### TABLE 1

**Effect of rapamycin on Ca^{2+}-sensitivity of isometric force**

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<td>1 µM</td>
<td>0.89 ± 0.04</td>
<td>2.77 ± 0.47</td>
<td>3.91 ± 0.58*</td>
<td>5.77 ± 0.01</td>
</tr>
<tr>
<td><strong>Rabbit cardiac</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 µM</td>
<td>0.97 ± 0.02</td>
<td>3.69 ± 0.29</td>
<td>3.05 ± 0.18*</td>
<td>5.55 ± 0.02</td>
</tr>
<tr>
<td><strong>Rat cardiac</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 µM</td>
<td>0.99 ± 0.07</td>
<td>3.76 ± 0.84</td>
<td>3.36 ± 0.59</td>
<td>5.40 ± 0.04</td>
</tr>
</tbody>
</table>

Isometric force is expressed as a ratio normalized to the pre-rapamycin control force for each individual muscle type. #Values (mean ± SEM; N = 6 for rabbit skeletal and cardiac, N = 8 for rat cardiac) were obtained from Hill fits to data from individual muscle preparations. Significance is expressed as * p < 0.05, when compared to 0 µM rapamycin.
Figure 1

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Rapamycin (M) 10^{-8} 10^{-7} 10^{-6} 10^{-5} 10^{-4} Normalized Force 0.0 0.2 0.4 0.6 0.8 1.0 1.2 Rat Cardiac Rabbit Cardiac Rabbit Psoas

Figure 2
Figure 3

A - Rabbit psoas

B - Rabbit Cardiac

C - Rat Cardiac

Normalized Force

pCa 5 6 7 8 9

0.0 0.2 0.4 0.6 0.8 1.0
Figure 4

A - Rabbit psoas

B - Rabbit Cardiac

Normalized $k_{TR}$ vs. Normalized Force

- pre-rapamycin
- rapamycin 1 µM
- post-rapamycin
Figure 5

Average Speed (µm s⁻¹)

[Rapamycin] (M)

0.0  0.5  1.0  1.5  2.0  2.5  3.0

0  0.5  1.0  1.5  2.0  2.5  3.0

*  **  **  **

10⁻⁹  10⁻⁸  10⁻⁷  10⁻⁶  10⁻⁵  10⁻⁴  10⁻³