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

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ABSTRACT

The immunosuppressant drug rapamycin attenuates the effects of many cardiac hypertrophy stimuli both in vitro and in vivo. Although rapamycin’s inhibition of mammalian target of rapamycin 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 antihypertrophic 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, whereas 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 actin filament sliding speed (~11%) in motility assays but inhibited sliding at 10⁻³ to 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 antihypertrophic activity being mediated through direct effects on myofilament contractility.

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 antirejection 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 translation processes that are central to cell growth (Thomas and Hall, 1997; Schmelze and Hall, 2000). mTOR exerts its translational control partially through activation of p70 ribosomal S6 kinase, or S6K1 (Schmelze and Hall, 2000). Rapamycin has been shown to be a strong inhibitor of S6K1 activation, presumably through its inhibition of mTOR (Chou and Bennis, 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 antihypertrophic activity in aortic banded mice is consistent with inhibition of the PI3K pathway (mTOR) causing suppression of load-in-

ABBRVIATIONS: FK506, tacrolimus; FKBP12, FK506-binding protein; PI3K, phosphoinositide 3-kinase; S6K1, p70 ribosomal S6 kinase 1; mTOR, mammalian target of rapamycin; FHCC, familial hypertrophic cardiomyopathy; F-actin, filamentous actin; L_s, initial sarcomere length; DTT, dithiothreitol; k_TR, kinetics of isometric tension redevelopment; HMM, heavy meromyosin; RhPh, rhodamine phalloidin; V_s, mean filament sliding speed.

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duced S6K1 activation (Shioi et al., 2003). Studies with dominant-negative PI3K 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). Although it remains possible that mTOR activation in these studies occurs through PI3K-independent pathways, alternative routes for rapamycin's antihypertrophic activity should be considered.

Binding of rapamycin to FKBP12 also alters Ca\textsuperscript{2+} transients in myocytes by dissociating FKBP12 from ryanodine receptors. Interestingly, this alteration differs between rabbit and mouse ventricular myocytes; rapamycin increases the Ca\textsuperscript{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\textsuperscript{2+}-calmodulin-dependent Ser/Thr phosphatase that alters nuclear factor of activated T-cells-dependent gene transcription and has been shown to participate in aortic banding-induced cardiac hypertrophy (Freys and Olson, 2003; Wilkins et al., 2004). Thus, rapamycin could influence the calcineurin-dependent hypertrophic response by altering Ca\textsuperscript{2+} release and, therefore, intracellular Ca\textsuperscript{2+}. There is, however, at least one additional possibility that may plausibly explain rapamycin's antihypertrophic 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\textsuperscript{2+} regulatory proteins tropomysin, 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 antihypertrophic 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\textsuperscript{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 antihypertrophic activity was mediated in part by direct effects on myofilament proteins, we predicted that rapamycin should have effects opposite those of FHC mutations: rapamycin would reduce force and/or sliding speed at a given [Ca\textsuperscript{2+}].

In the present study, we explored the acute effects of rapamycin on isometric force and Ca\textsuperscript{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\textsuperscript{2+}-activated force and inhibits F-actin sliding speed only at high concentrations. At concentrations where rapamycin affected submaximal force, it increased Ca\textsuperscript{2+} sensitivity. These results suggest that rapamycin's antihypertrophic effects do not involve direct inhibition of the myofilament proteins.

Materials and Methods

Animal Use

Animal handling was in accordance with the current National Institutes of Health/National Research Council 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.

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 the experiments were supplied by Sigma-Aldrich (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\textsubscript{2}O with 1 mg/ml sodium 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 = 3.3 kHz; Aurora Scientific Inc.) at the other end. The motor and force transducer were set to a modified stage of an inverted microscope (Chase and Kushmerick, 1988).

Initial sarcomere length (L\textsubscript{s}) (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 L\textsubscript{s} was set to 2.2 μm at pCa 9.0; skeletal fiber L\textsubscript{s} 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, 1 mM Pi, 10 mM EGTA, 50 mM MOPS, 45 mM free Na\textsuperscript{+}, 100 mM free K\textsuperscript{+}, 1 mM DTT, 200 to 400 units/ml creatine phosphokinase, and 4% w/v Dextran T-500 (Fisher Scientific Co., Pittsburgh, PA). Ca\textsuperscript{2+} solutions were prepared at evenly spaced pCa values (pCa = log[Ca\textsuperscript{2+}], where [Ca\textsuperscript{2+}] is in molar) ranging from pCa 9 (relaxing) to pCa 4.5 (maximally activating). To alter solution [Ca\textsuperscript{2+}], appropriate amounts of Ca(acetate)\textsubscript{2} were added, taking into account desired free [Ca\textsuperscript{2+}] and the binding constants of all solutions constituents for Ca\textsuperscript{2+}. Ionic strength was maintained constant (180 mM) by varying Tris and acetate. Solution pH was adjusted to 7.0 at 12°C. Rapamycin (R-5000; LC Laboratories, Woburn, MA) 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^{-5} M to 10^{-3} M. Ethanol (1%) 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 (Quart 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 \((k_{TR} = r^{-1} = -\ln 0.5 \times (t_{1/2})^{-1})\) (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:

\[
\frac{F}{F_0} = \frac{1}{1 + 10^{\frac{(pCa - pCa_{1/2})}{nH}}} \tag{1}
\]

where \(F_0\) is the maximum force at pCa 5, pCa\(_{50}\) is the pCa at which \(F = F_0/2\), and \(nH\) determines the steepness of the curve.

### 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 \(\mu\)g/ml glucose oxidase, 18 \(\mu\)g/ml catalase ( Worthington Biochemicals, Freehold, NJ), 0.3% methyl cellulose, and an additional 40 mM DTT were added to actin buffer, composed of 25 mM KCl, 25 mM imidazole, 4 mM MgCl\(_2\), 1 mM EGTA, and 1 mM DTT, pH 7.4 (Kron et al., 1991). Glucose, glucose oxidase, catalase, and extra DTT were added to minimize photobleaching 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\(^{-8}\) to 10\(^{-4}\) M, with a final concentration of 1% ethanol in all solutions as described for permeabilized muscle preparations. The negative control actin buffer (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 100× magnification, imaging six to eight fields from varying areas on the flow cell. Field images were recorded as 30-s clips using a VE1000 SIT camera (Dage-MTI, 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 Aasbury 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 S.D./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., Chicago, IL). Student’s \(t\) test was used to compare means.

### Results

**Maximum Ca\(^{2+}\)-Activated Force.** To determine whether rapamycin affects actomyosin interactions, we first measured maximum Ca\(^{2+}\)-activated (pCa 5) steady-state isometric force \((F_0)\) 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\(^{-8}\)–10\(^{-4}\) M) on maximum Ca\(^{2+}\)-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\(^{2+}\) activation: 1 \(\mu\)M, an effective immunosuppressant therapeutic dosage for mice that is comparable with human dosage (Shioi et al., 2003), and 100 \(\mu\)M, a maximum rapamycin concentration.

**Submaximal Ca\(^{2+}\) Activation of Isometric Force and Kinetics of Tension Redevelopment.** To determine the effects of rapamycin on Ca\(^{2+}\) 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 or 100 \mu 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 \mu M rapamycin increased Ca\textsuperscript{2+} sensitivity by 0.11 pCa units (Fig. 3C; Table 1), whereas there was no effect at 1 \mu M (data not shown). In rabbit cardiac and skeletal muscle, 1 \mu M rapamycin increased Ca\textsuperscript{2+} sensitivity by 0.08 or 0.13 pCa units, respectively (Fig. 3, A and B; Table 1); interestingly, there was no effect at 100 \mu M 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 1.67 ± 0.13 s\textsuperscript{-1} for rabbit cardiac muscle. \(k_{TR}\) increased nonlinearity 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 \mu M 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%, whereas 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 whether rapamycin affects unloaded sliding speed of F-actin in the absence of calcium regulatory proteins. This method 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 F-actin sliding speed (Fig. 5). At 10\textsuperscript{-7} M rapamycin, \(V_f\) increased by ~11% over the control (no rapamycin). At concentrations of 10\textsuperscript{-5} M or greater, there was a clear inhibition (60–80%) of \(V_f\) (Fig. 5).

Discussion

The purpose of the present investigation was to determine whether the beneficial effects of the antigrowth 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 antihypertrophic 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 1 week following aortic banding-induced hypertrophy, rapamycin had little or no effect on hearts from animals exhibiting compensated hypertrophy, whereas 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 vitro motility assay was utilized to determine whether rapamycin affects crossbridge cycling under unloaded conditions. The small but significant enhancement of V\(_f\) at \(10^{-7}\) M rapamycin and substantial inhibition at \(10^{-8}\) 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).

**TABLE 1**

<table>
<thead>
<tr>
<th>Muscle Type</th>
<th>Normalized (F_C)</th>
<th>Rapamycin Effect</th>
<th>Hill Coefficient (n_H)</th>
<th>(p_{Ca_{50}})</th>
<th>(\Delta p_{Ca_{50}})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit skeletal (1 (\mu)M)</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>
<td>5.90 ± 0.02</td>
</tr>
<tr>
<td>Rabbit cardiac (1 (\mu)M)</td>
<td>0.97 ± 0.02</td>
<td>3.89 ± 0.29</td>
<td>3.05 ± 0.18</td>
<td>6.05 ± 0.02</td>
<td>6.53 ± 0.01</td>
</tr>
<tr>
<td>Rat cardiac (100 (\mu)M)</td>
<td>0.89 ± 0.07</td>
<td>3.76 ± 0.84</td>
<td>3.36 ± 0.59</td>
<td>4.04 ± 0.04</td>
<td>3.51 ± 0.05</td>
</tr>
</tbody>
</table>

*Values (mean ± S.E.M.; n = 6 for rabbit skeletal and cardiac, n = 8 for rat cardiac) obtained from Hill fits to data from individual muscle preparations.

Significance expressed as: *p < 0.05; and **p < 0.01, when compared with the averaged control speed. Motility assays were conducted with F-actin and HMM from rabbit skeletal muscle.

![Fig. 4. The effects of rapamycin on activation dependence of \(k_{TR}\). Each point represents the averaged normalized \(k_{TR}\) for all muscle preparations analyzed at the given normalized force. Error bars represent S.E. 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.](image1)

![Fig. 5. Effects of rapamycin on unloaded sliding speed of RhPh-labeled F-actin in the in vitro motility assay. Speed is the weighted average of two flow cells per rapamycin concentration with an average of 160 filaments per flow cell. Error bars represent S.E. 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 with the averaged control speed. Motility assays were conducted with F-actin and HMM from rabbit skeletal muscle.](image2)
Ca²⁺ 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; Table 1) and the Ca²⁺ dependence of $k_{TR}$ (Fig. 4) in the presence and absence of rapamycin. This is particularly relevant because cardiac muscle functions at submaximum Ca²⁺ levels. Thus rapamycin could significantly influence cardiac physiology if it modulates function of the Ca²⁺ regulatory proteins, even though there was little or no effect at saturating Ca²⁺. Rabbit psoas and cardiac preparations showed an increase in Ca²⁺ sensitivity of isometric force at 1 μM rapamycin (Fig. 3; Table 1). There was no effect on the force-pCa relation in either rabbit preparation at a higher dose (100 μM) (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; Table 1). These steady-state force data suggest an influence of rapamycin on the thin-filament Ca²⁺ 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 Ca²⁺ in rabbit psoas fibers (Fig. 4A). $k_{TR}$ measured at submaximum Ca²⁺ is an indicator of the dynamics of thin filament regulatory function (Regnier et al., 1996, 1998); thus, the data in Fig. 4A indicate that rapamycin influences the Ca²⁺-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 Ca²⁺ dissociation from TnC or to differences in coupling between Ca²⁺ dissociation from TnC and strong crossbridge dissociation from actin (Landesberg and Sideman, 1994; Hancock et al., 1996; Regnier et al., 1996, 1998). Rapamycin’s inhibition of the $k_{TR}$-force relation in cardiac muscle was less 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 Antihypertrophic 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 mechanosensitive 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 dominant-negative PI3K 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.

Ca²⁺ 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 Ca²⁺ transients (Su et al., 2003). This alteration could also play a part in the antihypertrophic effects seen with rapamycin treatment and is consistent with the observed up-regulation of calcineurin/nuclear factor of activated T-cells 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 Ca²⁺ sensitivity in some aspect of cardiac function (Köhler et al., 2003; Wang et al., 2004). If increased Ca²⁺ sensitivity is associated with increased affinity of Ca²⁺ binding to TnC, then these mutations could also alter Ca²⁺ transients, which would then alter the Ca²⁺-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.

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References


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