2-Deoxyadenosine triphosphate restores the contractile function of cardiac myofibril from adult dogs with naturally occurring dilated cardiomyopathy

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Cheng Y, Hogarth KA, O’Sullivan ML, Regnier M, Pyle WG. 2-Deoxyadenosine triphosphate restores the contractile function of cardiac myofibril from adult dogs with naturally occurring dilated cardiomyopathy. Am J Physiol Heart Circ Physiol 310: H80–H91, 2016. First published October 23, 2015; doi:10.1152/ajpheart.00530.2015.—Dilated cardiomyopathy (DCM) is a major type of heart failure resulting from loss of systolic function. Naturally occurring canine DCM is a widely accepted experimental paradigm for studying human DCM. 2-Deoxyadenosine triphosphate (dATP) can be used by myosin and is a superior energy substrate over ATP for cross-bridge formation and increased systolic function. The objective of this study was to evaluate the beneficial effect of dATP on contractile function of cardiac myofibrils from dogs with naturally occurring DCM. We measured actomyosin NTPase activity and contraction/relaxation properties of isolated myofibrils from nonfailing (NF) and DCM canine hearts. NTPase assays indicated replacement of ATP with dATP significantly increased myofilament activity in both NF and DCM samples. dATP significantly improved maximal tension of DCM myofibrils to the NF level. Similarly, dATP increased the kinetics of contractile activation (kACT) with no impact on the rate of cross-bridge tension redevelopment (kTR). Thus, the activation kinetics (kACT/kTR) that were reduced in DCM samples were restored for NF sample levels. dATP also restored Ca2+ sensitivity of tension that was reduced in DCM samples. The rate of early slow-phase relaxation was slightly reduced with dATP, but its duration was not, nor was the fast-phase relaxation or times to 50 and 90% relaxation. Our findings suggest that myosin utilization of dATP improves cardiac myofibril contractile properties of naturally occurring DCM canine samples, restoring them to NF levels, without compromising relaxation. This suggests elevation of cardiac dATP is a promising approach for the treatment of DCM.

NEW & NOTEWORTHY

1) First report to characterize and define the contractile kinetics and defects associated with naturally occurring dilated cardiomyopathy (DCM) in dogs. 2) Novel findings that dATP is able to reverse the contractile defects associated with naturally occurring canine DCM.

Heart failure is the leading cause of mortality and morbidity in the United States, and it has been rising dramatically around the world each year (48a). Dilated cardiomyopathy (DCM) is a major type of heart failure (the prevalence is at least 1 in 2,500 individuals) that is characterized by dilatation and a diminished contractile function (reduced ejection fraction and cardiac output) of the left or both ventricles (18). The abnormally functioning heart triggers complex neurohormonal responses to maintain cardiac output through increased circulatory volume (18). The end stage of DCM is associated with the loss of systolic function, ventricular wall thinning, and myocardial fibrosis. The cause of a substantial percentage of DCM cases remains unknown, and no gene-specific therapy is yet available. Hence, it is of urgency to develop novel agents that can improve contractility and systolic function.

Several types of large breed dogs exhibit unusually high rates of familial DCM, including Irish Wolfhounds, Boxers, and Newfoundlands (43, 46, 47). Doberman Pinschers in particular present with a high occurrence of DCM, with studies reporting that up to 60% of the breed suffers from DCM (47). The genetic basis of canine DCM is not known despite several studies that have investigated this area (8, 24, 27, 39) and may in fact be breed specific. A mutation in the gene encoding for pyruvate dehydrogenase kinase 4 was identified as a putative cause for Doberman Pinschers (23), but this finding has been disputed (28), and a recent study has pointed to the potential for a polygenic basis of DCM in this breed (38). Despite the controversies involving the genetic cause of familial DCM in dogs, there exists a significant opportunity in studying the mechanistic foundation of canine DCM and exploring its therapeutic management. Similarities between the cardiovascular systems of humans and dogs allow for comparative investigations that can advance the understanding of this fatal condition. Furthermore, studies that use dogs that spontaneously develop DCM as opposed to the artificial induction of the disease allow for an investigation of heart failure in a naturally occurring entity that recapitulates key components of the innate condition.

We have previously reported that replacing ATP with 2-deoxyadenosine triphosphate (dATP) as an energy substrate improves contractility of striated muscle by enhancing cross-bridge binding/cycling kinetics and improving allosteric contractile activation (33–35, 37), and that increasing dATP level from the typical <0.1% of the adenosine nucleotide pool to ~1% significantly improves contraction (13). Increasing dATP levels in intact cardiomyocytes via adenovirus-mediated transfection of ribonucleotide reductase (R1R2) increases the magnitude and kinetics of contraction, and transgenic overexpressing R1R2 mice exhibit enhanced left ventricular (LV) basal systolic function compared with the control group (26). Recently, we applied dATP to cardiac tissue from end-stage heart failure human samples (25) and found that dATP increased force development and Ca2+ sensitivity of force. Additionally, isolated myofibrils from these samples had increased the force

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and rate of activation ($k_{ACT}$) with dATP, without altering relaxation (25).

We hypothesized that cardiac myofilaments from dogs with naturally occurring DCM would exhibit defects in myofibrillar kinetics that are normalized with exposure to dATP. Here, we report for the first time the myofibril contraction/relaxation kinetics for both nonfailing (NF) and naturally occurring DCM dog samples, as well as the ability of dATP to improve contraction/relaxation performance (of DCM) back to NF levels. Compared with NF samples, DCM samples had a reduced maximum tension production ($T_{MAX}$), Ca$^{2+}$ sensitivity of tension ($pCa_{50}$), and increased kinetics of tension redevelopment ($k_{TR}$). The rate of the early, slow-phase relaxation ($k_{REL,slow}$) was faster for the DCM myofibrils, but there was no difference in the larger, fast phase of relaxation ($k_{REL,fast}$). When ATP was replaced with dATP, $T_{MAX}$ and Ca$^{2+}$ sensitivity of tension were significantly improved for the DCM canine myofibrils, restoring them to almost the NF sample levels. dATP also significantly increased contractile $k_{ACT}$, with no impact on $k_{TR}$. dATP slightly reduced the rate of slow-phase relaxation but did not affect its duration, the fast-phase of relaxation, or times to 50 and 90% relaxation. Taken together, our results suggest that activation-relaxation kinetics are significantly impaired in naturally occurring canine DCM and that dATP improves myofibril contraction without impairing relaxation. dATP has merit as a promising therapeutic approach worth further investigating for the treatment of end-stage DCM.

**MATERIALS AND METHODS**

*Ethical approval and dog left ventricular tissue collection.* Animals were cared for in accordance with the guidelines of the Animal Care and Use Committee of the University of Guelph and the Canadian Council on Animal Care. Dogs were evaluated at the Ontario Veterinary College and at veterinary facilities in southern Ontario. Various breeds of dogs were included in the study (Doberman Pinscher, Mastiff, Boxer, Labrador Retriever) as were mixed-bred dogs. All dogs were classified as large breed, and body weights did not differ between NF controls and DCM (Table 1). NF controls were assessed by physical examination to rule out signs of cardiovascular disease. Postmortem histology and gross pathology were also used to rule out cardiovascular disease. DCM diagnosis was made on the basis of echocardiography (NF fractional shortening 24.0 ± 7.2%; DCM fractional shortening 7.7 ± 4.7%, $P < 0.05$) and confirmed with gross pathology and histopathology. Congestive heart failure (CHF) was determined on the basis of history, physical examination, and thoracic radiographs. Tissue samples were obtained from the LV free wall of dogs with advanced DCM causing CHF whose owners were electing humane death, or dogs with no previous history of cardiovascular disease (NF controls). Samples were rapidly frozen in liquid nitrogen and stored at −80°C for measurements of NT-Pase and contractile mechanical analysis. Written consent was obtained from clients for all procedures, including for the collection of myocardial samples.

*Solutions.* Composition of solution used for mechanical measurements was determined by an iterative algorithm that computes the equilibrium concentration of ions and ligands based on published affinity constants (5). Composition of muscle solution was as follows (in mM): 80 MOPS, 1 Mg$^{2+}$, 5 MgATP, 83 K$^+$, 52 Na$^+$, 15 EGTA, 15 creatine phosphate (CP), and 20 U/ml creatine phosphokinase, pH 7.0. The solution ionic strength was 170 mM, and the Inorganic phosphate (Pi) concentration determined by nuclear magnetic resonance (NMR) measurement was 0.5 mM (15). The Ca$^{2+}$ levels (expressed as pCa = −log([Ca$^{2+}$])) for activation solutions were adjusted by adding CaCl$_2$. For the ATP vs. dATP study, 2 mM energy substrate (ATP or dATP) was added to the muscle solution just before the experiment and was used up within the same day.

*Mycofilament mechanical and kinetics measurement.* Frozen tissues were shipped to the University of Washington Medical Campus on dry ice and stored at −80°C until used. A small piece of flash-frozen cardiac tissue from the LV free wall of canine hearts was thawed and demembranated overnight at 4°C in a solution containing 1% Triton detergent (by volume), 50% glycerol (by volume), 1:100 dilution “Protease-Inhibitor-Cocktail” (Sigma-Aldrich, St. Louis, MO), and a muscle relaxing solution, as previously described (3, 14, 37). Muscle bundles were rinsed two times in Rigor solution containing 1 mM DTT and 1:100 dilution of protease inhibitor before being homogenized for two pulses of 30 s on ice at high speed. Myofibrils were used the same day of preparation.

Myofibril mechanical/kinetics measurements were performed on a custom setup as previously described (15, 16, 31, 32). Briefly, single or small bundles (~2–4) of canine cardiac myofibrils were attached between two glass microtools with the sarcomere length set as ~2.3 μm and perfused with solutions that are rapidly switched. Activation and relaxation data were measured at 15°C and fit as previously described (15, 16, 31, 32). $k_{ACT}$ (with rapid increase in Ca$^{2+}$) was obtained from a single-exponential rise to a maximum. To measure the time course of force redevelopment, the release-restretch protocol was applied. A sudden decrease in length (20% of optimal length) was imposed on the myofibrils, and the myofibrils were then rapidly stretched back to their original length after 25 ms of unloaded shortening. $k_{REL,slow}$ was reported as the slope of a regression line fit to the tension trace and normalized to the tension amplitude, and the slow-phase duration ($t_{REL,slow}$) was measured from the onset of solution change at the myofibril to the shoulder marking the beginning of the fast phase. Transition from slow to rapid phase was determined through multiple factors. An apparent change in the slope of the data or a change in the signal-to-noise ratio was often apparent at the transition. $k_{REL,fast}$ was measured from a single-exponential decay fitted to the data. A half-time ($t_{1/2}$) estimation was made in cases where the decay was not well described by a single exponential, and this was converted to a rate $\tau$ = ln(2)/$t_{1/2}$. Myofibrils that contracted >10% of their optimal length were excluded from the analysis as nonsimetric.

*NT-Pase assays.* Cardiac myofilaments were prepared according to a modified protocol from Yang et al. (49). Briefly, hearts were homogenized in an ice-cold Standard Buffer and centrifuged at 12,000 g for 15 min at 4°C. The pellets were resuspended in ice-cold Standard Buffer containing 1% Triton X-100 and gently agitated at 4°C for 45 min. The suspension was washed three times (1,100 g for 15 min at 4°C) in ice-cold Standard Buffer. Protein concentration was measured with a Bradford Protein Assay (Bio-Rad Laboratories, Mississauga, ON). Actomyosin NT-Pase activity was determined using a modified Carter assay (49). Cardiac myofilaments (50 μg) were incubated in

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Table 1. **Echocardiographic parameters of hearts from nonfailing and dilated cardiomyopathic dogs**

<table>
<thead>
<tr>
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<th>Nonfailing</th>
<th>DCM</th>
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<tbody>
<tr>
<td>Body Wt, kg</td>
<td>36.1 ± 4.4</td>
<td>38.1 ± 6.2</td>
</tr>
<tr>
<td>Age, yr</td>
<td>5.6 ± 0.8</td>
<td>6.8 ± 1.3</td>
</tr>
<tr>
<td>Gender distribution</td>
<td>2 male, 4 female (1 intact in each group)</td>
<td>5 male, 1 female (all spayed or neutered)</td>
</tr>
<tr>
<td>Left ventricular end-diastolic diameter, mm</td>
<td>38.4 ± 2.3</td>
<td>60.4 ± 7.1*</td>
</tr>
<tr>
<td>Left ventricular end-systolic diameter, mm</td>
<td>29.2 ± 3.7</td>
<td>55.5 ± 6.2*</td>
</tr>
<tr>
<td>Fractional shortening, %</td>
<td>24.0 ± 7.2</td>
<td>7.7 ± 4.7*</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>113 ± 12</td>
<td>128 ± 27</td>
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Values are presented as means ± SE; $N$ = 6 hearts/group, DCM, dilated cardiomyopathy. *$P < 0.05$ vs. nonfailing.
reaction buffers containing variable free Ca\(^{2+}\) concentrations ([Ca\(^{2+}\)]\(_f\)) made by mixing activating and relaxing buffers. Free Ca\(^{2+}\) was calculated using the program from Patton et al. (29). Myofilaments were incubated in reaction buffers for 10 min at 32°C and quenched with 10% trichloroacetic acid. P1 production was measured following the addition of an equal volume of 0.5% FeSO\(_4\) and 0.5% ammonium molybdate in 0.5 M H\(_2\)SO\(_4\) and reading the absorbance at 630 nm.

Myofilament protein phosphorylation profile. Myofilament proteins (10 µg) were separated using 12% SDS-PAGE. Gels were fixed overnight in 50% methanol-10% acetic acid at room temperature. Gels were incubated for 2 h at room temperature with Pro-Q Diamond (Molecular Probes, Eugene, OR) to stain phosphorylated proteins. Gels were imaged with a Bio-Rad Chemi Doc MP Imaging System (Bio-Rad) and analyzed with ImageJ software (NIH, Bethesda, MD). Protein loading was assessed by Coomassie blue staining of gels after Pro-Q Diamond imaging.

Statistics. Comparison between groups of data was performed using paired or unpaired Student’s t-test as appropriate. All data are expressed as means ± SE, “n” represents the number of experimental samples in each group, and “N” represents the number of dogs in each group. Results with P < 0.05 were considered statistically significant.

RESULTS

Contractile properties of DCM and NF cardiac myofibrils. We compared tension development and the kinetics of activation and relaxation for small bundles (~2–4) of cardiac myofibrils from either DCM or NF canine samples. Mounted myofibrils were exposed to continually flowing solutions that were rapidly switched to produce step increases and decreases in bathing [Ca\(^{2+}\)] from relaxing solution (pCa 9.0) to either maximal (pCa 4.0) or submaximal (pCa 5.6, 5.8, and 6.0) [Ca\(^{2+}\)], then finally back to pCa 9.0. A pair of representative tension traces for NF vs. DCM myofibrils during the maximal [Ca\(^{2+}\)] activation-relaxation protocol is presented in Fig. 1. A summary of tension and kinetic parameters for both NF and DCM dog myofibrils is presented in Table 2 and Figs. 2 and 3. TMAX was ~35% lower for myofibrils obtained from DCM vs. NF dog samples (P < 0.005, Fig. 2A). Tension production was also collected at multiple submaximal Ca\(^{2+}\) levels, and tension was less at each pCa in DCM vs. NF samples (P < 0.005). The pC50\(_{0}\) was right shifted by 0.22 pCa units for DCM samples (Fig. 2B), from 5.99 ± 0.01 (NF samples, N = 52, n = 5) to 5.77 ± 0.01 (DCM samples, N = 45, n = 5, P < 0.001), indicating that DCM leads to a reduction of Ca\(^{2+}\) sensitivity of the contractile apparatus.

kACT following rapid switching of bathing solution from pCa 9.0 to 4.0 (or submaximal Ca\(^{2+}\) levels) includes the kinetic processes of Ca\(^{2+}\)-dependent thin filament activation, myosin cross-bridge binding, and the subsequent tension development. Compared with the NF samples, kACT did not differ for the DCM samples (P = 0.492) at pCa 4.0 or any submaximal Ca\(^{2+}\) level tested (Table 2). For both NF and DCM myofibrils, kACT was significantly slower during submaximal Ca\(^{2+}\) activations than at the maximal Ca\(^{2+}\) level, as previously reported for rodent cardiac myofibrils (15, 32), demonstrating that the Ca\(^{2+}\) sensitivity of the kinetics of cardiac contractile activation is maintained in dogs with DCM. Once activation was completed (i.e., tension was in steady state), a rapid release and restretch protocol was applied to measure kTR. The kTR protocol measures the rate of myosin cross-bridge attachment and subsequent tension generation (3) when Ca\(^{2+}\) binding to troponin is in near steady state (e.g., the thin filament is already activated).

A comparison of kACT with kTR can help to differentiate between the cross-bridge vs. Ca\(^{2+}\)-mediated thin filament activation contributions to the kinetics of tension generation. As we have previously reported for myofibrils from mouse (16) and rat (32) hearts, kTR was faster than kACT at all Ca\(^{2+}\) levels for both DCM and NF dog samples. This indicates that thin filament activation is rate limited for dog cardiac myofibrils by the kinetics of thin filament activation at 15°C. At pCa 4.0, kTR was ~70% faster for the DCM vs. NF samples (P < 0.005), and a significant difference was also detected at all submaximal Ca\(^{2+}\) conditions (Fig. 2D). Comparison of the kACT-to-kTR ratio gives an indication of whether thin filament activation kinetics are more rate limiting to tension development in DCM samples compared with NF myofibrils. Figure 2E demonstrates that this was indeed the case at pCa 4.0 (P < 0.005), as well as for all the submaximal Ca\(^{2+}\) levels (P < 0.005).

Rapid deactivation of myofibrils by a second switching of solutions from maximal or submaximal [Ca\(^{2+}\)] to relaxing solution (pCa 9.0) produced a biphasic relaxation, with an initial, small linear tension decay followed by a rapid exponential decay back to the baseline tension (see the inset of Fig. 1 for an example trace). Here, kREL,slow is considered to predominantly reflect the rate of cross-bridge detachment (7,
dATP restores contractile function of DCM cardiac myofibrils. We next investigated the effects of replacing ATP with dATP as the nucleotide substrate for contraction for all DCM canine samples. To control for the effects of repeated activations, myofibrils from each DCM canine were activated and relaxed at both maximal and submaximal Ca\textsuperscript{2+} levels with 2 mM ATP followed by 2 mM dATP in activation and relaxation solutions for one-half the preparations, and the reverse order was used for the other one-half. A pair of normalized example tension traces for DCM myofibrils treated with dATP, then ATP during the maximal [Ca\textsuperscript{2+}] activation-relaxation protocol, is shown in Fig. 4A, and the raw tension trace is shown in the inset. The tension magnitude and kinetic parameters for all DCM dog myofibrils (treated with 2 mM ATP or dATP) are summarized in Table 3 and Figs. 4 and 5. Compared with activation with ATP, the $T_{\text{MAX}}$ of DCM samples was increased -30% to 64.9 2.0 mN/mm\textsuperscript{2} with dATP ($P < 0.005$, Fig. 4B). This restored tension to near the level for the NF samples with ATP (67.2 2.2 mN/mm\textsuperscript{2}, $P = 0.4113$), as can be seen in the inset of Fig. 4A. dATP also significantly increased $k_{\text{ACT}}$ ($P < 0.005$, Fig. 4C) but had no impact on $k_{\text{TR}}$ ($P = 0.9272$, Fig. 4D). Thus, the ratio of $k_{\text{ACT}}/k_{\text{TR}}$ was increased (~35%, $P < 0.005$, Fig. 4E) with dATP to the level seen for NF samples (0.73 0.04, $P = 0.1749$). Interestingly, dATP slowed $k_{\text{REL,slow}}$ a small but statistically significant amount ($P < 0.005$, Fig. 5B) for DCM myofibrils, but there was no effect on $t_{\text{REL,slow}}$ ($P = 0.0625$, Fig. 5C). Representative tension traces demonstrating this are presented in Fig. 5A, which also shows that there were no major differences for ATP vs. dATP for $k_{\text{REL,fast}}$ ($P = 0.2975$, Fig. 5D), $RT_{50}$ ($P = 0.2565$, Fig. 5E), or $RT_{90}$ ($P = 0.2515$, Fig. 5F).

We also determined how tension and the kinetics of activation and relaxation were affected by dATP at submaximal Ca\textsuperscript{2+}, where the heart operates. Here, we chose a pCa level (pCa 5.8) that achieved approximately half-maximal (pCa\textsubscript{50}) activation of the DCM canine myofibrils in the presence of ATP. For the DCM myofibrils, dATP significantly increased tension from 53 to 64% of $T_{\text{MAX}}$ ($P < 0.005$), suggesting increased Ca\textsuperscript{2+} sensitivity of tension (Fig. 5B). dATP also increased $k_{\text{ACT}}$ ($P < 0.01$, Fig. 4C) but had no impact on $k_{\text{TR}}$ ($P = 0.7221$, Fig. 4D), increasing the ratio of $k_{\text{ACT}}/k_{\text{TR}}$ ($P < 0.005$, Fig. 4E) to that seen for NF samples. With dATP, relaxation at pCa 5.8 reduced $k_{\text{REL,slow}}$ ($P < 0.005$, Fig. 5B) compared with ATP, but $t_{\text{REL,slow}}$ ($P = 0.0623$, Fig. 5C) was not affected, nor was $k_{\text{REL,fast}}$ ($P = 0.0994$, Fig. 5D), $RT_{50}$ ($P = 0.1307$, Fig. 5E), or $RT_{90}$ ($P = 0.0577$, Fig. 5F). Thus both activation and relaxation of DCM samples were restored to NF sample levels with dATP. We also collected data on the activation/relaxation parameters of dATP vs. ATP for the NF dog samples, and the results are summarized in Table 4.
Fig. 2. Tension at multiple Ca$^{2+}$ levels (A), the pCa-tension relationship (B), $k_{ACT}$ (C), $k_{TR}$ (D), the ratio of $k_{ACT}/k_{TR}$ (E), and the $k_{TR}$-relative tension relationship (F) for both NF vs. DCM dog cardiac myofibrils. **$P < 0.005$.

Fig. 3. The rate ($k_{REL,\text{slow}}$, A) and duration ($t_{REL,\text{slow}}$, B) of slow-phase relaxation, and time to reach 50% (C) and 90% (D) relaxation for both NF vs. DCM dog cardiac myofibrils at multiple Ca$^{2+}$ levels. *$P < 0.05$ and **$P < 0.005$. 

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Similar to the DCM samples, dATP also significantly increased tension production and $k_{ACT}$ and had little effect on the relaxation for the NF samples.

**NTPase assays.** Cardiac myofilaments were isolated from NF and DCM myocardial samples, and actomyosin NTPase activity was assessed across a range of free $[Ca^{2+}]$ (Fig. 6A). Although there were no significant differences in maximum activation, myofilament $Ca^{2+}$ sensitivity as measured by $pCa_{50}$ was higher ($P = 0.032$) in DCM myofilaments compared with NF. Replacement of ATP with dATP increased actomyosin ATPase activity at both maximal and submaximal $[Ca^{2+}]$ in NF samples (Fig. 6A). Similarly, myofilaments from DCM samples exhibited a significant increase in activity across the range of $[Ca^{2+}]$ (Fig. 6A).

**Phosphorylation profile.** The phosphorylation status of key myofilament proteins was determined by staining SDS-PAGE separated proteins with Pro-Q Diamond phosphostain (Fig. 6B). In DCM myofilaments, desmin phosphorylation was increased by 53% over NF samples ($P = 0.01$), whereas troponin I phosphorylation declined by 23% ($P = 0.02$). Coomassie staining (Fig. 6C) showed no significant difference in the amounts of myofilament proteins loaded across lanes, although the band corresponding to desmin appeared to be increased. Exposure of the myofilaments to dATP did not alter phosphor-

**Fig. 4. A:** normalized tension trace for DCM dog cardiac myofibrils treated with ATP (black) vs. 2-deoxyadenosine triphosphate (dATP, red) during maximal $Ca^{2+}$ activation. The inset is a close up of activation with real tension [the tension ($B$), $k_{ACT}$ ($C$), $k_{TR}$ ($D$), and the ratio of $k_{ACT}/k_{TR}$ ($E$)] of DCM dog myofibrils treated with ATP vs. dATP at maximal ($pCa 4.0$) and submaximal ($pCa 5.8$) $Ca^{2+}$ levels. *$P < 0.05$ and **$P < 0.005$.**
Table 3. Tension generation and relaxation parameters for DCM dog myofibrils treated with ATP and dATP at 15°C

<table>
<thead>
<tr>
<th>Relaxation</th>
<th>Fast Phase</th>
<th>Slow Phase</th>
</tr>
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<tbody>
<tr>
<td>kREL,fast,s</td>
<td>1 kACT/s</td>
<td>1 kREL,slow,m</td>
</tr>
<tr>
<td>RT50,s</td>
<td>0.05 (82)</td>
<td>2.54 (77)</td>
</tr>
<tr>
<td>RT90,s</td>
<td>0.12 (49)</td>
<td>0.03** (74)</td>
</tr>
<tr>
<td>Tension, mN/mm²</td>
<td>5.8 28.3</td>
<td>4.0 64.9</td>
</tr>
</tbody>
</table>

Comparison of DCM vs. NF canine samples. DCM myofibrils produced less tension (compared with NF samples) during maximal Ca²⁺ activation (Fig. 2A) and had a reduced Ca²⁺ sensitivity of tension (Fig. 2B). Interestingly, maximal actomyosin MgNTPase was slowed by 10% for DCM samples (Fig. 6), but there was no difference in submaximal NTPase activity. This difference with respect to the tension data may be due to the different condition (loaded vs. unloaded) and the loss of constraints from the three-dimensional sarcomere structure in the NTPase assays. However, it may also be indicative of an inefficient system in which myofibrillar ATP consumption does not translate into an effective force-generating cross-bridge. Interestingly, Malik et al. (20) found that promoting the transition from weak to strong, force-generating crossbridges with omecamtiv mecarbil improved myocardial function in a canine model of heart failure, which would support the possible involvement of energetically inefficient crossbridges in the pathogenesis of DCM.

To understand how DCM may affect thin filament activation, we compared the rapid release-restretch protocol (kTR) with the Ca²⁺-activation protocol (kACT) during the same activation trial. The kACT/kTR ratio can act as a quantitative measure of the thin filament activation processes that must occur before myosin cross-bridge formation and during tension generation. Here, we found that kTR was faster than kACT for both NF and DCM canine cardiac myofibrils at all Ca²⁺ levels, indicating that thin filament activation was rate limiting for tension generation of the myofibrils at 15°C. This finding is similar to our previous work in rodent hearts (15, 32) but differs from several other reports that found no difference between kACT and kTR (30, 42). For the myofibril activation/relaxation measurements, the solutions were continually flowing to the mounted myofibrils such that there was a continual replenishing of nucleotide triphosphates, e.g., no reduction of the ATP or dATP concentration. Also, CP and creatine phosphokinase (20 U/ml) were included in activation solutions. With both ATP (5 mM) and CP (15 mM) in solutions, we normally see a natural contaminating amount of Pi, ~0.5 mM (periodically measured by NMR), that derives from the high-
energy phosphates in solutions (15). Other studies often use a phosphate “mop” to reduce this Pi in solutions. We choose to make our solutions more closely match the Pi composition found in heart muscle, which has a basal level of Pi. The presence of Pi specifically affects the cross-bridge tension-generating isomerization (increase that rate) without influencing thin filament activation kinetics, thus Pi affects kTR but has little effect of kACT. Our current findings confirm our previous results in rodents (15, 32), and extend them to a larger animal where H9252-myosin is the predominant myosin isoform, demonstrating a faster kTR (than kACT) at both maximal and submaximal levels of Ca2+ in both NF and DCM canine samples. By comparing the relationship between kTR and relative tension at a given [Ca2+] (Fig. 2F), our data indicated no Ca2+ or tension dependence of kTR in the NF dog heart samples, and only a slight but not significant (P = 0.0824) increase as tension rose from 30% (pCa 6.0) to 100% (pCa 4.0) maximal tension for the DCM dog heart samples. We (36) and others (48) have reported both Ca2+ and tension dependence of kTR in previous studies, although it is not as robust in large animals with predominant H9252-myosin. Thus it is unclear why we did not see it in the current studies.

In addition to kTR, kREL,slow was also faster for DCM canine cardiac myofibrils compared with the NF samples. In general, both kTR and kREL,slow are (in part) indicators of the cross-bridge cycling rate and/or the rate of cross-bridge detachment (30, 40, 42). However, it is still important to point out that the conditions for these two measurements are different. For instance, kTR is collected when Ca2+ in activation solutions is constant and Ca2+ binding to cardiac troponin (cTn) is in the steady state. In contrast, kREL,slow is measured following the abrupt removal of Ca2+ from the system. Therefore, the amount of Ca2+ bound to the thin filament is more dynamic and is reduced at a rate determined by Ca2+ dissociation rate of cTn. The change in bound Ca2+ is likely most dramatic at the beginning of the relaxation phase, when the gradient of Ca2+-bound (to cTn) vs. that in solution is highest. Thus, caution should be used when comparing kTR vs. kREL,slow as a measure of cross-bridge cycling or detachment kinetics. Nonetheless, our finding that both kTR and kREL,slow are altered in DCM myofibrils is indicative of changes in cross-bridge cycling in failing canine hearts. While we saw no significant increase in maximal ATPase in the DCM samples, the Ca2+ sensitivity of ATPase was elevated in correlation with the more rapid kinetics. This occurs even though there is a lower Ca2+ sensitivity of tension development, suggesting that submaximal contraction (where the heart operates in vivo) may be energetically less efficient.

Chronic changes in myofilament protein phosphorylation have emerged as potential players in the failing heart (9, 17). Within the myofilament complex there are several proteins that alter myofilament and cardiac function when their phosphorylation status is changed, and within many of these proteins there exist various phosphorylatable residues whose functional impact is profound and sometimes paradoxical. While some studies have demonstrated a link between specific phosphorylation changes and discrete functional outcomes, the reality of diseased hearts is that myofilament phosphorylation is altered in a complex manner that precludes a simple linear extrapolation to precise transformations of cardiac performance. Here we show that in end-stage canine DCM there is a significant increase in desmin phosphorylation along with declines in troponin I and myosin light chain-2 phosphorylation. Phosphorylated desmin is hypothesized to form amyloid-like complexes that disrupt sarcomeric integrity and tension development in failing hearts (1), a functional change that is consistent with our tension development data. Troponin I phosphorylation in failing hearts has been the subject of debate (21), but several


### Table 4. Tension generation and relaxation parameters for NF dog myofibrils treated with ATP and dATP at 15°C

<table>
<thead>
<tr>
<th>Myofibril</th>
<th>Batch no.</th>
<th>pCa</th>
<th>Tension, mN/m²</th>
<th>kACT</th>
<th>kTR</th>
<th>kREL.slow</th>
<th>kREL.slow, s</th>
<th>kREL.slow/m²</th>
<th>kREL.slow, s</th>
<th>kREL.slow/m²</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF with ATP</td>
<td>(ω = 5)</td>
<td>4.0</td>
<td>64.5 ± 2.7 (37)</td>
<td>1.29 ± 0.07 (37)</td>
<td>1.26 ± 0.06 (37)</td>
<td>0.03 ± 0.00 (37)</td>
<td>0.02 ± 0.00 (37)</td>
<td>0.03 ± 0.00 (37)</td>
<td>0.02 ± 0.00 (37)</td>
<td></td>
</tr>
<tr>
<td>NF with dATP</td>
<td>(ω = 5)</td>
<td>4.0</td>
<td>73.8 ± 3.6 (33)</td>
<td>1.63 ± 0.11 (25)</td>
<td>1.27 ± 0.12 (25)</td>
<td>0.15 ± 0.01 (33)</td>
<td>0.02 ± 0.00 (26)</td>
<td>0.02 ± 0.00 (26)</td>
<td>0.02 ± 0.00 (26)</td>
<td></td>
</tr>
<tr>
<td>NF with dATP</td>
<td>(ω = 25)</td>
<td>4.0</td>
<td>35.1 ± 2.6 (28)</td>
<td>1.10 ± 0.08 (25)</td>
<td>0.57 ± 0.02 (27)</td>
<td>0.02 ± 0.00 (26)</td>
<td>0.02 ± 0.00 (26)</td>
<td>0.02 ± 0.00 (26)</td>
<td>0.02 ± 0.00 (26)</td>
<td></td>
</tr>
</tbody>
</table>

Values given are means ± SE; n, no. of batches. Nos. in parentheses are no. of myofibrils. *p < 0.05 vs. ATP.

### Beneficial effects of dATP on DCM cardiac myofibrils.

Our previous studies with rodent models demonstrated that the magnitude and rate of force development of cardiac muscle was increased when dATP is substituted for ATP (13, 26, 34, 37). Recently, we reported this is also true for myocardium from human end-stage heart failure patients (25). Here we report a similar finding for myocardium from NF and DCM dogs, demonstrating that dATP increases the magnitude and rate of tension generation in myocardium irrespective of the predominant myosin isoform. Although the canine model is widely accepted as an experimental paradigm for studying human DCM, in part because the dominant myosin isoform in canine is also a β-myosin heavy chain, previous studies using dogs have typically relied on pacing to induce heart failure in otherwise healthy animals. To our knowledge, this is the first study to measure changes in myofilament kinetics in naturally occurring canine DCM and to evaluate the effect of dATP on the contraction of naturally occurring DCM canine samples. Our NTPase assays indicate that dATP increased the actomyosin MgATPase activity at both saturating and submaximal free Ca²⁺ levels for both NF (p < 0.05) and DCM (p < 0.05) canine samples. These results are consistent with our kinetic measurements on both NF and DCM canine myofibrils with dATP vs. ATP in which dATP significantly elevated tension and kACT but did not elevate kTR and actually slowed kREL.slow. All of the above findings suggest that dATP may improve the efficiency of contraction. Schoffstall et al. reported that porcine cardiac preparations had a similar dATP-induced increase in maximal force production but did not show a change in Ca²⁺ sensitivity of force, a result that was attributed to troponin and tropomyosin isoform differences (11). Interestingly, we recently reported a gene therapy approach that results in in-
creased cardiac dATP which significantly improved ejection fraction, left ventricular pressure development, the rate of pressure development, and pressure loss in pigs treated 2 wk after myocardial infarct (11).

**Clinical application/future direction.** The results from our current study have potential implications for treating DCM, resulting from the thinning ventricular walls and loss of systolic function. Our findings suggest that myosin utilization of dATP instead of ATP as a contractile substrate improves contractile properties of cardiac myofibrils from naturally occurring DCM canine samples, without compromising relaxation, and elevation of cardiac dATP is a promising approach for treatment of DCM.

The net tension gained with replacement of ATP with dATP is almost the same for myocardium from mice, rats, dogs, pigs, and humans. Thus, this current report for canine myofibrils (a clinical model for heart failure), along with the pig infarct (11) and failing human myocardium (25) studies, suggests elevating dATP in the myocardium may be an effective approach for treatment of many forms of systolic heart failure.

Unlike our previous finding that dATP has no impact on the relaxation (25), here we found that the rate of the early slow-phase relaxation was reduced somewhat with dATP vs. ATP. However, neither the duration of slow-phase nor the fast-phase relaxation was affected. Considering the very small contribution of slow-phase relaxation (5% of total relaxation), this should have very little impact on relaxation. To confirm this, we further calculated RT50 and RT90. Our results demonstrated that there were no major increases in the ability of DCM canine myofibrils to relax with dATP vs. ATP. This is
H90  dATP AND CANINE DCM

important, since it demonstrates that systolic performance of DCM cardiac muscle is improved with dATP without affecting the early phase of diastole associated with ventricular relaxation.

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GRANTS

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DISCLOSURES

MR is a co-founder and equity holder in BEAT Biotherapeutics, which has licensed his technology to treat heart failure via elevation of dATP in myocardium from the University of Washington.

AUTHOR CONTRIBUTIONS


REFERENCES


