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Original article

Upregulation of cardiomyocyte ribonucleotide reductase increases intracellular 2 deoxy-ATP, contractility, and relaxation

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ABSTRACT

We have previously demonstrated that substitution of ATP with 2 deoxy-ATP (dATP) increased the magnitude and rate of force production at all levels of Ca^{2+}-mediated activation in demembranated cardiac muscle. In the current study we hypothesized that cellular [dATP] could be increased by viral-mediated overexpression of the ribonucleotide reductase (Rrm1 and Rrm2) complex, which would increase contractility of adult rat cardiomyocytes. Cell length and ratiometric (Fura2) Ca^{2+} fluorescence were monitored by video microscopy. At 0.5 Hz stimulation, the extent of shortening was increased ~40% and maximal rate of shortening was increased ~80% in cardiomyocytes overexpressing Rrm1 + Rrm2 as compared to non-transduced cardiomyocytes. The maximal rate of relaxation was also increased ~150% with Rrm1 + Rrm2 overexpression, resulting in decreased time to 50% relaxation over non-transduced cardiomyocytes. These differences were even more dramatic when compared to cardiomyocytes expressing GFP-only. Interestingly, Rrm1 + Rrm2 overexpression had no effect on minimal or maximal intracellular [Ca^{2+}], indicating increased contractility is primarily due to increased myofilament activity without altering Ca^{2+} release from the sarcoplasmic reticulum. Additionally, functional potentiation was maintained with Rrm1 + Rrm2 overexpression as stimulation frequency was increased (1 Hz and 2 Hz). HPLC analysis indicated cellular [dATP] was increased by approximately 10-fold following transduction, becoming ~1.5% of the adenine nucleotide pool. Furthermore, 2% dATP was sufficient to significantly increase crossbridge binding and contractile force during sub-maximal Ca^{2+} activation in demembranated cardiac muscle. These experiments demonstrate the feasibility of directly targeting the actin–myosin chemomechanical crossbridge cycle to enhance cardiac contractility and relaxation without affecting minimal or maximal Ca^{2+}. This article is part of a Special issue entitled "Possible Editorial".

1. Introduction

Systolic and/or diastolic cardiac function is compromised in a number of cardiovascular diseases including myocardial infarction, ischemia/reperfusion injury, diabetes, high blood pressure and hypertrophic and dilated cardiomyopathy. These pathophysiological conditions often involve alterations in the Ca^{2+} cycle [1], β-adrenergic responsiveness [2], and/or the contractile apparatus of cardiomyocytes [3,4]. To date, therapeutic efforts have focused primarily on approaches that increase $[Ca^{2+}]_i$ that can be pro-arrhythmogenic and may impair ventricular filling by slowing diastolic relaxation [5]. Other approaches involving adrenergic agents can have undesirable long-term consequences, including significant side-effects due to drug actions in non-target areas, pro-arrythmogenic triggered activity, and potential for accelerated progression into heart failure [2]. Thus, new approaches to combat cardiac dysfunction are desirable.

We have previously shown that replacing ATP with 2 deoxy-ATP (dATP) as the substrate for contraction of demembranated cardiac muscle increased isometric force and the rate of force development and shortening at all levels of Ca^{2+} activation, including saturating [Ca^{2+}] (pCa 4.0) [6–9]. The presence of dATP results in enhanced myosin binding to actin and an increase in the rates of P_i and dADP release and myosin detachments. As such, contractile properties can be improved by >50% over the range of $[Ca^{2+}]_i$ seen *in vivo*. Thus, replacement of ATP with dATP offers the potential to improve contraction independent of changes in $[Ca^{2+}]_i$ or adrenergic signaling.

Abbreviations: Rrm1, muscle ribonucleotide reductase 1; Rrm2, muscle ribonucleotide reductase 2; ARC, adult rat cardiomyocyte; GFP, green fluorescent protein; RT_{50}, RT_{90}, time to 50% and 90% relaxation; DT_{50}, DT_{90}, time to 50% and 90% Ca^{2+} decay.

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To date, the effect of dATP has only been studied in demembranated cardiac tissue and with isolated contractile proteins. As such, its potential to improve intact cardiomyocyte contraction or cardiac function *in situ* is unknown. Cellular production of dATP occurs in the cytoplasm of mammalian cells by ribonucleotide reductase (Rrm), which removes a hydroxyl moiety from the 2-position on the ribose ring of ADP to produce dADP. dADP is then rapidly converted to dATP. Rrm consists of two subunit proteins, a catalytic activator (Rrm1) and free radical containing (Rrm2) subunit and is regulated by nucleoside triphosphate allosteric effectors [10]. While the details of regulating cellular RR content, enzymatic activity and cellular concentration [dATP] are unclear, it is known that both subunits are necessary for activity [11].

In the current study, we produced adenoviral vectors expressing cytomegalovirus (CMV) promoter driven Rrm1 or Rrm2, each along with green fluorescent protein (GFP) as a transduction reporter. Cultured adult rat cardiomyocytes were transduced with these vectors, and the rate and extent of myocyte contraction and relaxation and Ca^{2+} transient rise and decay (Fura2 fluorescence) were monitored by video microscopy following a 48 h viral incubation period. Here we show that these treatments significantly increased cellular [dATP], rate and extent of shortening, and rate of relaxation, with minimal effects on Ca^{2+} transients, at 0.5 Hz, 1 Hz and 2 Hz stimulation. Additionally, the [dATP] found in transduced cells (1–2% of adenine nucleotide content) was sufficient to increase sub-maximal Ca^{2+} activated force in skinned cardiac trabeculae. These experiments suggest that increases in cardiac intracellular Rrm and/or the dATP pool can significantly alter the actin–myosin crossbridge cycle to enhance cardiac contractility without impairing diastolic function or cardiomyocyte Ca^{2+} handling.

2. Methods

Greater details of plasmid design and vector production, cell culture, contractile assessment, nucleotide binding affinity, and western blot analysis are provided in online supporting information.

2.1. Animal and tissue handling

These studies were approved by the University of Washington (UW) Animal Care Committee and conducted in accordance with federal guidelines. Animals were cared for in accordance with US NIH Policy on Humane Care and Use of Laboratory Animals in the Department of Comparative Medicine at UW. Adult rat (Fischer 344) cardiomyocytes (ARCs) were isolated from heart using aortic retrograde perfusion for enzymatic (collagenase/protease) dispersion of cells [12]. Neonatal Rat Cardiomyocytes (NRCs) were isolated by enzymatic dispersion from 1 to 3-day old newborn Fischer 344 rats as previously described [13]. Rat cardiac trabeculae were dissected from the right ventricle of male Sprague–Dawley rats, chemically demembranated, and prepared for mechanical measurements as previously described [13]. Trabeculae averaged 1.3 ± 0.2 mm in length by 170 ± 30 μm in width.

2.2. Plasmid design and virus production

HEK293 cells were used to generate adenoviral vectors [14] expressing Rrm1 or Rrm2 from the CMV promotor. Both vectors contained a second expression cassette for green fluorescent protein (GFP) as a transduction reporter protein, and we also expressed a vector for GFP-only. Virus was introduced to cardiomyocytes at ~250 particles per cell.

2.3. Nucleotide binding affinity

Rapid kinetic measurements of nucleotide binding and actin–myosin dissociation were taken at 10 °C and 20 °C (Hi-Tech Scientific SF-61 DX2 stopped-flow system) as previously described [15] using pyrene labeled actin and myosin S1. Myosin was purified from mouse hearts, rabbit soleus, and rabbit bulk fast muscle as previously described [16,17]. Actin was purified from rabbit skeletal muscle [18]. The stopped-flow transients were fitted to one or two exponentials by non-linear least squares curve fitting using the Kinetic Studio software (TgK Scientific). All experiments were carried out in 20 mM Cacodylate buffer, pH 7.0 containing 100 mM KCl, and 5 mM $MgCl_2$. The rate constant for ATP-induced actin-S1 dissociation (k_{obs}) was determined from Eq. (1) based on SI Scheme 1:

$$k_{obs} = k_{+2}K_1 \frac{[ATP]}{1 + K_1[ATP]} \quad (1)$$

2.4. Contractile assessments

In modified Tyrodes buffer at ambient temperature (22–24 °C) and at 37 °C, cell shortening and relaxation of arbitrarily selected stimulated cardiomyocytes was recorded using IonOptix system video microscopy. (IonOptix, Milton, MA, USA). Calcium transients induced by electrical stimulation were measured in Fura2 loaded cells using IonOptix equipment as described [19]. Fura2 fluorescence was measured using an IonOptix spectrophotometer (Stepper Switch) attached to a fluorescence microscope. Emitted Fura2 fluorescence was collected by the 40X objective, passed through a 510 nm filter and detected by a photomultiplier tube. For demembranated trabeculae, steady-state force and high frequency sinusoidal stiffness (to determine crossbridge binding) were measured in a custom built mechanical apparatus at 15° and 22 °C during sub-maximal (pCa 5.6) and maximal (pCa 4.0) Ca^{2+} activation as previously described [20]. Experimental physiological Ca^{2+} solutions were calculated as previously described for trabeculae mechanics [21].

2.5. Data processing and statistical analysis

Maximal cardiomyocyte shortening and relengthening and calcium transient rise and decay were calculated offline using IonOptix software to determine the maximum of the first derivative of these transients. Times to peak shortening and 50% and 90% return to baseline were also calculated offline. Statistical differences were determined by ANOVA, with Student-Newman–Keuls as a *post-hoc* pairwise test (SigmaPlot 11). Trabeculae were compared using paired t-tests. Differences at p-value < 0.05 were considered statistically significant. Data is displayed as mean ± s.e.m.

3. Results

Transduction with recombinant adenovirus containing appropriate cDNA constructs driven by the CMV promoter was used to induce overexpression of muscle ribonucleotide reductase 1 (Rrm1) and 2 (Rrm2) in cultured adult and neonatal rat cardiomyocytes. Each adenovirus also contained a second expression cassette for green fluorescent protein (GFP), which was used as a reporter protein identifying successful transduction. Cardiomyocytes were infected with adenovirus containing genes for [Rrm1 + GFP and Rrm2 + GFP] or [GFP] for 2 days. Successful gene transfer, grossly indicated by green fluorescence with microscopy, indicated nearly 100% transduction efficiency (Supplemental Fig. 1). This is consistent with previous studies using cardiomyocytes [22]. Cell survival over this period was similar for all groups, including non-transduced control cells, suggesting that these viral vectors did not compromise cardiomyocyte viability. Cardiomyocyte numbers and sarcomere lengths are summarized in Table 1. There was no difference in resting sarcomere length between groups, indicating that overexpression of Rrm1 + Rrm2 (or GFP) did not increase calcium independent activation.