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Synergistic role of ADP and Ca^{2+} in diastolic myocardial stiffness

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Running title: Cross-bridging the gap between energetics and Ca^{2+}

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Key points:

- Diastolic dysfunction in heart failure patients is evident from stiffening of the passive properties of the ventricular wall.
- Increased actomyosin interactions may significantly limit diastolic capacity, however direct evidence is absent.
- From experiments at the cellular and whole organ level, in humans and rats, we show that actomyosin-related force development contributes significantly to high diastolic stiffness in environments where high ADP and increased diastolic $[Ca^{2+}]$ are present, such as the failing myocardium.
- Our basal study provides a mechanical mechanism which may partly underlie diastolic dysfunction.

Abstract

Heart failure (HF) with diastolic dysfunction has been attributed to increased myocardial stiffness that limits proper filling of the ventricle. Altered cross-bridge interaction may significantly contribute to high diastolic stiffness, but this has not been shown thus far. Cross-bridge interactions are dependent on cytosolic $[Ca^{2+}]$ and the regeneration of ATP from ADP. Depletion of myocardial energy reserve is a hallmark of HF leading to ADP accumulation and disturbed Ca^{2+}-handling. Here, we investigated if ADP elevation in concert with increased diastolic $[Ca^{2+}]$ promotes diastolic cross-bridge formation and force generation and thereby increases diastolic stiffness. ADP dose-dependently increased force production in the absence of Ca^{2+} in membrane-permeabilized cardiomyocytes from human hearts. Moreover, physiological levels of ADP increased actomyosin force generation in the presence of Ca^{2+} both in human and rat membrane-permeabilized cardiomyocytes. Diastolic stress measured at physiological lattice spacing and 37°C in the presence of pathological levels of ADP and diastolic $[Ca^{2+}]$ revealed a 76±1% contribution of cross-bridge interaction to total diastolic stress in rat membrane-permeabilized cardiomyocytes. Inhibition of creatine kinase (CK), which increases cytosolic ADP, in enzyme-isolated intact rat cardiomyocytes impaired diastolic re-lengthening associated with diastolic Ca^{2+}-overload. In isolated Langendorff-perfused rat hearts, CK-inhibition increased ventricular stiffness only in the presence of diastolic $[Ca^{2+}]$. We propose that elevations of intracellular ADP in specific types of cardiac disease, including those where myocardial energy reserve is limited, contribute to diastolic dysfunction by recruiting cross-bridges even at low Ca^{2+} and thereby increase myocardial stiffness.

Abbreviations

HF, Heart failure; CK, Creatine kinase; PCr, Phosphocreatine; LVEDP, Left ventricle end-diastolic pressure; IDCM, idiopathic dilated cardiomyopathy; F_{pas}, Passive tension; ADP-F_{total}, ADP-stimulated total tension; ADP-F_{act}, ADP-active tension; Ca^{2+}-F_{total}, Ca^{2+}-stimulated total tension; Ca^{2+}-F_{act}, Ca^{2+}-active tension; EC_{50}, ADP- or Ca^{2+}-sensitivity (at which half of F_{act} is reached); ktr, Ca^{2+}-ADP dependent rate of force redevelopment; PV, Pressure-volume; DNFB, 2,4-dinitro-1-fluorobenzene; SERCA, Sarcoplasmic reticulum Ca^{2+}-ATPase

Introduction

Heart failure (HF) is a clinical syndrome defined as the inability of the heart to sufficiently supply blood to organs and tissues (McMurray & Pfeffer, 2005). While contractile dysfunction is present in about half of the HF patient population almost all patients show impaired diastolic function (McMurray & Pfeffer, 2005; Borlaug & Paulus, 2011). Pathophysiological mechanisms which have been related with diastolic dysfunction involve alterations of the passive properties of the ventricular wall, including high titin-based myofilament passive stiffness and collagen deposition, and slow rates of myocardial relaxation due to increased myofilament Ca^{2+}-sensitivity and impaired Ca^{2+}-reuptake (Leite-Moreira, 2006; van Heerebeek *et al.*, 2012; Hamdani & Paulus, 2013).

Depletion of myocardial energy reserve represents a major cause of dysfunction of the failing human heart (Neubauer, 2007). In the healthy heart, creatine kinase (CK) catalyzes the transfer of phosphate from phosphocreatine (PCr) to ADP, thereby regenerating ATP, while preventing accumulation of cytosolic ADP (Allen & Orchard, 1987). ATP is essential for cross-bridge cycling. As little as 0.1 mM ATP is sufficient for cross-bridge detachment and sarcomere relaxation (Cooke & Bialek, 1979). The relatively high myocardial *in vivo* ATP levels in both healthy (8-11 mM) and various pathological conditions (7-10 mM) (Tian *et al.*, 1997b; Spindler *et al.*, 1998; He *et al.*, 2007) indicate that reductions in ATP levels are unlikely to directly impair relaxation of the heart muscle. Notably, HF patients with diastolic dysfunction (Smith *et al.*, 2006; Phan *et al.*, 2009) and animal models of HF (Hamman *et al.*, 1995; Spindler *et al.*, 1998; Horn *et al.*, 2001; He *et al.*, 2007) show that the reduced PCr/ATP ratio (a parameter of myocardial energy reserve measured *in vivo*) is mostly explained by a severe reduction in PCr (up to 50%) rather than changes of ATP (≤25%). Reducing PCr or experimental inhibition of CK activity is causally linked to the development of HF, as it elevates left ventricle end-diastolic pressure (LVEDP), reduces contractile reserve and increases mortality in rats (Hamman *et al.*, 1995; Tian *et al.*, 1997a; Horn *et al.*, 2001). Activity of CK isoforms is decreased by 50% in animal models of HF (Khuchua *et al.*, 1989; Nascimben *et al.*, 1995; Neubauer *et al.*, 1995). The consequence of low PCr and/or a reduced CK activity is that cytosolic levels of ADP will increase. Indeed, increases in [ADP] of >50% have been reported in several

animal models of HF (Tian *et al.*, 1997b; Spindler *et al.*, 1998; He *et al.*, 2007). Selectively increasing ADP levels without altering cytosolic ATP levels has been shown to increase LVEDP and limit myocardial relaxation in rats (Tian *et al.*, 1997a; Tian *et al.*, 1997b). These studies support the idea that limited myocardial energy reserve via elevations of ADP may represent a likely cause of diastolic dysfunction. However the exact pathophysiological mechanism has not been elucidated.

Intriguingly, ADP can stimulate tension generation, in the absence of Ca^{2+}, in membrane-permeabilized rabbit skeletal muscle fibers (Shimizu *et al.*, 1992) and bovine cardiac muscle (Fukuda *et al.*, 1996; Fukuda *et al.*, 1998). These findings indicate that ADP accumulation, as a consequence of disturbed myocardial energy reserve, may lead to incomplete relaxation during diastole caused by residual actomyosin interactions. However, in HF, the accumulation of ADP occurs concomitantly with disturbed Ca^{2+}-handling (Beuckelmann *et al.*, 1992; Shannon *et al.*, 2003; Undrovinas *et al.*, 2010). The effects of elevated intracellular ADP and high Ca^{2+} levels on human myofilament function that exist during the diastolic phase are currently not known. We hypothesize that in failing hearts enhanced cross-bridge formation and force generation, as a result of increased levels of ADP and diastolic $[Ca^{2+}]$ increase diastolic stiffness.

Methods

Ethical Approval

Human samples were obtained after written informed consent and with approval of St Vincent's Hospital (#H03/118) and from the Human Research Ethics Committee of the University of Sydney (#159401). The investigation conforms with the principles outlined in the Declaration of Helsinki (1997). All animal experiments were performed with approval of the Animal Care and Use Committee of the VU University Medical Center (Amsterdam) and UK Home Office and local ethical review.

Myocardial human samples

Human left ventricular (LV) tissue was obtained during heart transplantion surgey from end-stage failing patients with idiopathic dilated cardiomyopathy (IDCM). Tissue was immediately frozen and stored in liquid nitrogen.

Isometric force measurements in membrane-permeabilized single human cardiomyocytes

Small human cardiac tissue samples (N=3) were thawed in relaxing solution and cardiomyocytes were mechanically isolated by tissue disruption as described previously (van der Velden *et al.*, 1998). Cardiomyocytes were chemically permeabilized by incubation for 5 minutes in relaxing solution containing 0.5% (v/v) Triton-X100 and glued between a force transducer and a piezoelectric motor. Isometric force measurements were subsequently performed on single cardiomyocytes at a sarcomere length of 2.2 μm. Absolute force values were normalized to cardiomyocyte cross-sectional area and expressed as developed tension (in kN/m^2). Passive tension (F_{pas}) was determined by allowing the cardiomyocyte to shorten by 30% of its length in relaxing solution. ADP-stimulated total tension was determined by activating the cardiomyocyte at 10 mM ADP (ADP-F_{total}, Figure 1A). Similarly, Ca^{2+}-stimulated total tension was determined by activating the cardiomyocyte at 32 μM Ca^{2+} (Ca^{2+}-F_{total}; Figure 1A). Active tension (Ca^{2+}-F_{act} or ADP-F_{act}) was calculated as $F_{act}=F_{total}-F_{pas}$. In addition, the rate of force

redevelopment (ktr) was determined using a slack-restretch test as follows: after steady state force was reached, cardiomyocytes were allowed to shorten to 70% of their original length within 1 ms and then re-stretched to the original length after 30 ms. A single exponential was fitted to determine the rate constant of force redevelopment: ktr was determined at maximal and submaximal $[Ca^{2+}]$ in the presence of ADP to assess cross-bridge cycling kinetics.

Experimental solutions

The composition of all solutions was calculated based on a computer program similar to that described previously (Papp *et al.*, 2002). The pH of all solutions was adjusted to 7.1 at 15ºC with KOH and ionic strength was adjusted to 180 mM with KCl. The relaxing solution contained 2 mM free Mg^{2+}, 1 mM MgATP, 20 mM EGTA, 10 mM BES and 14.5 mM PCr (P7936, Sigma). Several activating solutions were prepared: 1) Ca^{2+}-activating solution consisted of 2 mM free Mg^{2+}, 1 mM MgATP, 20 mM EGTA, 10 mM BES and 32 μM free Ca^{2+}. Ca^{2+}-activating solutions with lower free $[Ca^{2+}]$ were obtained by mixing of the Ca^{2+}-activating and relaxing solutions and assuming an apparent stability constant of the Ca^{2+} -EGTA complex of $10^{6.35}$; 2) ADP-activating solutions (in the absence of free Ca^{2+}) contained 2 mM free Mg^{2+}, 1 mM MgATP, 20 mM EGTA, 10 mM BES and MgADP in increasing concentrations from 1 to 10 mM; 3) Ca^{2+}- activating solutions in the presence of ADP consisted of 2 mM free Mg^{2+}, 1 mM MgATP, 20 mM EGTA, 10 mM BES, MgADP concentrations from 0.02 to 6 mM and free Ca^{2+} from 1 to 32 μM. PCr was omitted from the activating solutions to preserve the MgADP/MgATP ratio. All force measurements were performed at 15ºC. [ATP] was maintained constant at 1 mM to avoid rigor cross-bridge formation (reported to occur when [ATP] falls below 0.1 mM (Cooke & Bialek, 1979)) and consecutively increased the [ADP] from 1 to 10 mM. We used a maximum [ADP] of 10 mM, since ADP-stimulated tension was maximal at 10 mM ADP with 1 mM ATP in bovine cardiac muscle (Fukuda *et al.*, 1996; Fukuda *et al.*, 1998).

Data analysis of isometric force measurements

Ca^{2+}- and/or ADP-force relations were determined by a non-linear fit procedure using a modified Hill equation using KaleidaGraph version 3.6 (Synergy Software, Reading, PA) as follows accordingly:

$$P\,(Ca^{2+})\,/\,P_0 = [Ca^{2+\ nH}\,/\,(K^{nH} + [Ca^{2+}]^{nH})$$

$$P\,(ADP)\,/\,P_0 = [ADP]^{nH}\,/\,(K^{nH} + [ADP]^{nH})$$

$$P\,(Ca^{2+}\text{-}ADP)\,/\,P_0 = [Ca^{2+}\text{-}ADP]^{nH}\,/\,(K^{nH} + [Ca^{2+}\text{-}ADP]^{nH})$$

where P is steady-state force. P_0 denotes the steady isometric force at either saturating $[Ca^{2+}]$, high [ADP] or both saturating $[Ca^{2+}]$ and high [ADP], nH describes the steepness of the relationship, and K represents the $[Ca^{2+}]$ and/or [ADP] at which force is half-maximal ($0.5 \times P_0$). Myofilament Ca^{2+}- and/or ADP-sensitivity is denoted as EC_{50}.

Isolation of intact rat ventricular cardiomyocytes

Intact rat cardiomyocytes were isolated using collagenase digestion of hearts as described previously (King *et al.*, 2011). Briefly, adult wild-type male Wistar rats (N=7) weighing ~300 grams were euthanized with isoflurane and hearts were quickly removed and rinsed in cold isolation-Tyrode solution (composition: 130 mM NaCl, 5.4 mM KCl, 3 mM NaPyruvate, 25 mM HEPES, 0.5 mM $MgCl_2$, 0.33 mM NaH_2PO4, 22 mM glucose) containing 0.2 mM EGTA (Tyrode-EGTA) and pH 7.4. The heart was then cannulated via the aorta to the Langendorff apparatus and perfused for 2 min with Tyrode-EGTA at 37°C. Thereafter, the heart was perfused with enzyme-Tyrode solution consisting of Tyrode solution, 1.2 mg/ml collagenase (Type II, 265 U/mg; Worthington Biochemical, NJ, USA) and 50 μM $CaCl_2$ for a period of 7 min. The right ventricle and atria were removed, and the LV was cut into small pieces and triturated with a plastic Pasteur pipette for 3 min in stopping buffer solution 1 (SB-1; composition: Tyrode solution, 0.6 mg/ml collagenase, 100 μM $CaCl_2$ and 10 mg/ml bovine serum albumin (BSA)). The cell suspension was

filtered through a 300 μm nylon mesh filter into a 50 ml Falcon tube and centrifuged for 1 min at 27x g (20-23ºC). The pellet containing cardiomyocytes was resuspended in SB solution 2 (SB-2; composition: Tyrode solution, 250 μM $CaCl_2$ and 10 mg/ml BSA) and incubated for 10 min in a water bath at 37ºC allowing cells to settle. The supernatant was discarded and cardiomyocytes were resuspended in SB solution 3 (SB-3; containing Tyrode solution, 500 μM $CaCl_2$ and 10 mg/ml BSA), incubated for 10 min at 37ºC and subsequently resuspended in experimental Tyrode (used for measurements of intact cardiomyocytes), consisting of 1.2 mM $CaCl_2$, 137 mM NaCl, 5.4 mM KCl, 0.33 mM NaH_2PO4, 0.57 mM $MgCl_2$, 5 mM HEPES and 5.6 mM glucose. Cardiomyocytes were used within the first 6h of isolation.

Isometric force measurements in membrane-permeabilized single rat cardiomyocytes
Single cardiomyocytes isolated by collagenase digestion from adult wild-type male Wistar rats (N=4) were used. Cardiomyocytes were chemically permeabilized with 0.5% (v/v) Triton-X100 as indicated above for human and isometric force measurements were performed at sarcomere lengths of 2.2 μm. Experimental temperature and solution conditions were similar as previously described for human samples. Ca^{2+}-force relations without and with ADP, and the rate of cross-bridge cycling was determined as previously described for human samples.

Diastolic stress measurements in membrane-permeabilized rat cardiomyocytes
Single cardiomyocytes isolated by collagenase digestion from adult wild-type male Wistar rats (N=5) were used. Cardiomyocytes were chemically permeabilized with 0.5% (v/v) Triton-X100 as indicated above and isometric force measurements were performed in single cardiomyocytes stretched from 1.8 to 2.4 μm sarcomere lengths. Experiments were performed at 15ºC to replicate conditions currently employed to study diastolic stiffness/stress and at a higher, more physiological temperature of 37ºC. Since membrane-permeabilization causes myofilament lattice expansion, experiments were also performed with the osmotic agent dextran T500 (4%, w/v) that compresses the myofilament lattice to physiological levels (Irving *et al.*, 2000). Additionally, 30 μM of the cross-bridge inhibitor

blebbistatin was used to assess the contribution of cross-bridge formation to passive stress. Force generation was normalized to cardiomyocyte cross-sectional area and expressed as diastolic stress (in kN/m^2). The composition and characterization of all 4 conditions is displayed in Table 1. To mimic conditions currently in use to study diastolic stiffness/stress, experiments were performed at 15ºC in the 1) absence and 2) presence of blebbistatin. To approach *in vivo* disease conditions measurements were performed at 37ºC and with Dextran T500 in the 3) absence and 4) presence of blebbistatin. Because blebbistatin is photosensitive, experiments were performed in the dark. To ensure whether total absolute tension values were absent of protocol artefacts due to substantial differences in cross-sectional area that could artificially increase or decrease diastolic stress, all cross-sectional measurements were done in solutions without dextran. Cross-sectional area did not statistically differ between conditions 1 to 4 (342.8±18, 370.1±39, 354±22 and 350±36 $kN/m^2/\mu m$, respectively).

Cardiomyocyte shortening and Ca²⁺-transient of intact rat cardiomyocytes

Cardiomyocyte shortening and Ca^{2+}-transient of intact rat cardiomyocytes
Unloaded shortening and Ca^{2+}-transients of intact rat cardiomyocytes (n=15 to 20) were monitored following field stimulation (1Hz, 4 ms, 7V), and sarcomere shortening as well as relaxation kinetics were visualized using a video-based sarcomere length (SL) detection system (IonOptix corporation). Wild-type rat cardiomyocytes were incubated in Tyrode solution containing 1 µM Fura-2-AM for 15 min and rinsed for a period of 10 min in Tyrode. Cardiomyocytes were placed into a temperature-controlled (37ºC) chamber with platinum electrodes to electrically stimulate cells. Single cardiomyocytes were randomly selected (without spontaneous contraction) and continuously perfused with Tyrode buffer. After 3-5 min field stimulation sarcomere shortening as well as relaxation kinetics and Ca^{2+}-transient were monitored. Cardiomyocytes loaded with Fura-2-AM were excited at 340 and 380 nm with emission monitored at 510 nm. The F340/F380 ratio was used as a measure of cytosolic [Ca^{2+}]. From the monitored Ca^{2+}-transient, Ca^{2+}-release and -reuptake velocity, the time to reach peak systolic Ca^{2+} and the time constant of Ca^{2+} decline (tau) were determined.

Experimental protocol for unloaded rat cardiomyocyte measurements

To test the hypothesis that ADP accumulation impairs myofilament function in cardiomyocytes with intact membrane, a protocol was established using a low dose of an irreversible inhibitor of CK, iodoacetamide. Specifically, 5 mM fresh iodoacetamide (I6125, Sigma) was dissolved in Tyrode buffer and perfused (at 1 ml/min) for 3 min, delivering a total dose of 15 μmoles/3 ml. This drug has well-characterized effects on the ATP-regeneration system, such that it significantly increases [ADP] without altering ATP, PCr, inorganic phosphate and pH levels in isolated perfused hearts (Tian *et al.*, 1997a; Tian *et al.*, 1997b). ADP is estimated to be lower than 73 μM at this dose range (Tian *et al.*, 1997a).

Two protocols were used to test the effects of CK-inhibition (Fig. 5). Cardiomyocytes were paced at 1Hz for 3-5 min with continuous perfusion with Tyrode solution (at 1 ml/min and 36±0.1°C) to establish a steady state. Thereafter, a number of cells was either perfused for 3 min with a total dose of 15 μmoles/3 ml of iodoacetamide (Fig. 5A) or continuously perfused with Tyrode solution (Fig. 5C). Following 3 min treatment with iodoacetamide, perfusion with Tyrode solution was restored. Perfusion was limited to 3 min/ml with 5 mM of iodoacetamide, because the Ca^{2+}-Fura signal deteriorated by longer exposure and/or greater concentration of drug. Control experiments to check for Ca^{2+}-fluorescence demonstrated that iodoacetamide does not quench Ca^{2+} (data not shown). Measurements were averaged and analyzed in three phases as detailed in the legend of Figure 5. The control protocol was the same as the treatment protocol except that cells were continuously perfused with Tyrode solution.

Isolated perfused Langendorff hearts: Diastolic pressure-volume relationships

Adult wild-type male Wistar rats were euthanized by stunning and cervical dislocation and hearts quickly removed and rinsed in cold Krebs-Henseleit (KH) solution. Diastolic pressure-volume relationships were measured in hearts connected to a Langendorff apparatus and perfused with Ca^{2+}-free KH solution gassed with 95% O_2-5% CO_2 at 37°C and pH 7.4. An inflatable balloon (filled with pre-boiled, degassed Milli-Q water) was placed in the left ventricle and the volume adjusted with a syringe pump dispensing at 400

µL/min. Balloon volume was inferred from dispensing rate and pressure was recorded continuously via a side port connected to a pressure transducer. Balloon volume was increased in cycles to elicit a series of pressures from 10-30 mmHg allowing the balloon to conform to the LV cavity surface; the final cycle to 30 mmHg was repeated and all diastolic pressure-volume data were obtained from it (LeGrice *et al.*, 2012).

The experimental procedures were as follows: diastolic pressure-volume relationship of Langendorff-perfused rat hearts were recorded after 60 min perfusion with Ca^{2+}-free KH with (N=5 hearts) or without (N=6 hearts) 20 µM of the cell-permeant Ca^{2+}-chelator BAPTA-AM (Cambridge Bioscience, UK) dissolved in DMSO (final concentration <0.1%). Perfusion solution was then switched to a Ca^{2+}-free KH containing 20 µM 2,4-dinitro-1-fluorobenzene (DNFB; 42085, Sigma) dissolved in ethanol (final concentration <0.1%), for 30 min then PV measurements were repeated. DNFB is an irreversible inhibitor of CK which decreases the ATP/ADP ratio (Gercken & Schlette, 1968). Because iodoacetamide inhibits enzymes of the glycolytic reaction (even though well-oxygenated hearts have normal glycolysis after iodoacetamide-treatment (Tian *et al.*, 1997a)), whereas DNFB does not (Westerblad & Lännergren, 1995), we wanted to ensure complete preservation of the glycolytic flux following long chemical inhibition of a multicellular system such as the whole heart. DNFB has been shown to produce similar effects in isolated cardiomyocytes as iodoacetamide (Fowler *et al.*, 2014).

Creatine Kinase activity

CK activity levels were assessed using a commercial colorimetric assay kit (ab155901, Abcam) that detects kinase activity as low as 1 mU/ml (nmol/min/ml). Activity was assessed in freshly isolated cell suspensions from rat cardiomyocytes (N=3) as follows: from each single animal, two suspensions of isolated cells were treated (i.e. one treated with buffer (control) and another with 5 mM iodoacetamide) for 5 minutes, centrifuged for 30 seconds at 27x g (37ºC) and supernatant discarded. The pellet containing cardiomyocytes was immediately frozen in liquid nitrogen and freeze-dried overnight. Assay was performed as specified by manufacturer supplied information. Similarly, CK

activity was evaluated in fresh rat cells from 3 animals without and in the presence of 20 μM of DNFB.

Statistical analysis

Data analysis and statistics were performed using Prism version 6.0 (Graphpad Software, Inc., La Jolla, CA) and SAS/STAT® 9.2 (Statistical Analysis System Software, Institute Inc., Cary, NC, USA).

Data are presented as mean ± SEM of all single cardiomyocytes per patient/animal or group. All data was tested for normality using the Shapiro-Wilk Test. Normality was assumed when p>0.05 and the variances were equal. Paired- and/or unpaired-sample Student's t-Test was conducted when one variable was tested between 2 groups of data. Single classification analysis of variance (ANOVA) was performed when one variable was tested in more than 2 groups. When a significant difference was found in repeated measures 1-way ANOVA, Bonferroni *post hoc* testing was used to determine the location of significant differences. When more than one variable was tested in more than 2 groups a 2-way ANOVA was conducted followed by a Bonferroni *post hoc* test if significant differences were found. A linear mixed model was performed to gain insights into the individual trajectories of repeated measurements of each data group (e.g. intercepts and slopes) compared with controls. This model takes into account the repeated measurements within subjects. Each performed test is specified in Tables or Figure legends. The level of significance was set at p<0.05 and exact significance level (p values) are given in Supporting Tables 1 and 2.

Results

ADP stimulates myofilament contraction in the absence of Ca^{2+}

To assess whether ADP alone can induce contraction in human cardiac muscle, force produced by single membrane-permeabilized cardiomyocytes from human failing hearts was measured at increasing [ADP]. ADP caused force production in the absence of Ca^{2+} with maximal tension (ADP-F$_{act}$) achieved with 10 mM ADP and tension reaching 80±3% of maximal Ca^{2+}-developed tension (Fig. 1A). Comparable to Ca^{2+}, force-ADP relation curves show the common sigmoidal pattern, indicating cooperative activation (Fig. 1B). ADP-induced tension at 1 mM ADP amounted to 14±1% and nearly saturated at 8 mM ADP (94±3%). Half-maximal ADP-F$_{act}$ (EC$_{50}$) was 3.80±0.15 mM ADP (Fig. 1B).

ADP increases myofilament Ca^{2+}-sensitivity

Myocardial ADP levels in animal models are reported in the range of 10-50 μM in health and of 40-140 μM in disease (Tian *et al.*, 1997b; Spindler *et al.*, 1998; He *et al.*, 2007), indicative that *in vivo* levels of ADP are incapable of myocardial activation since *in vitro* mM levels of ADP are required for force generation (Fig. 1B). Cardiomyocytes *in vivo* are activated by the cytosolic Ca^{2+} transient that varies from ~0.15 μM during diastole to a peak of ~1.6 μM in systole (Bers, 2002). We therefore investigated if elevation of ADP in concert with physiological levels of Ca^{2+} increases force generation in human membrane-permeabilized cardiomyocytes (Fig. 2A). Physiological levels of ADP were chosen in the range of 20 and 100 μM. ADP shifted the Ca^{2+}-force relation substantially to the left, indicative of increased Ca^{2+}-sensitization of myofilaments (Fig. 2A). Indeed, as little as 20 μM ADP significantly increased Ca^{2+}-F$_{act}$ generation, evident from the elevated Ca^{2+}-sensitivity compared with the Ca^{2+}-force relation without ADP (EC$_{50}$=1.69±0.10 and 1.95±0.09 μM Ca^{2+}, respectively). Our data suggests that conditions that promote elevation of myocardial ADP result in cross-bridge recruitment even at low Ca^{2+}, supporting delayed muscle relaxation.

ADP slows cross-bridge detachment in the presence of Ca^{2+}

The rate of myofilament contraction and relaxation is regulated by the rate of attachment and detachment of force producing cross-bridges, i.e. cross-bridge cycling. Therefore, the rate of force redevelopment (ktr, a measure of the rate of cross-bridge cycling) in the presence of Ca^{2+} and ADP was examined, and values were plotted against the corresponding isometric force (Fig. 2B). When Ca^{2+}-activated isometric force rises with increasing [ADP] the ktr relationships shifted substantially to the right and downwards with significant differences found for relationships with supra-physiological ADP levels present ($\geq$2 mM ADP; Supp Table 1). This suggests that force increases at the expense of slowing cross-bridge cycling (Fig. 2B), likely resulting from a population of strongly-bound cross-bridges with slow detachment kinetics. This is consistent with significant increases in sarcomere stiffness (i.e. high residual force recovery) at [ADP] $\geq$20 µM, indicative for reduced actomyosin detachment and enhanced strain/force per each individual cross-bridge (Figs. 2C and 2D).

To test whether the latter changes in myofilament Ca^{2+}-sensitivity, cross-bridge cycling and residual force recovery (Fig. 2) are species-independent, similar experiments were performed in rat membrane-permeabilized cardiomyocytes in the presence of 100 µM ADP. Rat membrane-permeabilized cardiomyocytes present similar dependence to ADP as humans, such as increased myofilament Ca^{2+}-sensitization, slower rates of cross-bridge cycling and high residual force recovery (Fig. 3).

Together these data indicate that in membrane-permeabilized cardiomyocytes the combined presence of Ca^{2+} and physiological ADP levels increases cross-bridge stiffness, even at low Ca^{2+} levels, which could contribute to high sarcomere stiffness and reduce LV compliance during the diastolic phase.

Re-evaluation of the influence of cross-bridges on in vitro diastolic stress with in vivo conditions

In the last decade, numerous *in vitro* studies assessed the diastolic properties of human HF myocardium and no evidence was found for the possible contribution of cross-bridges to the high diastolic stiffness observed in disease (van Heerebeek *et al.*, 2012; Hamdani &

Paulus, 2013). Nevertheless, the majority of the studies thus far have been based on membrane-permeabilized myocytes, where the relatively low experimental temperature (15ºC) in concert with sarcolemma digestion that causes lattice expansion and removes important differences in intracellular milieu (such as increased ADP levels), may largely underestimate cross-bridge interactions. To overcome some of these concerns we adjusted the protocol previously reported by King *et al.*(King *et al.*, 2011), where diastolic stress was measured in rat membrane-permeabilized cardiomyocytes at 37ºC in the presence of the osmotic agent dextran to compress myofilament lattice restoring it to physiological conditions. By measuring cardiomyocyte diastolic stress, in the presence of diastolic $[Ca^{2+}]$ and [ADP] known to occur in disease with and without the cross-bridge inhibitor blebbistatin (30 μM), we were able to assess the contribution of cross-bridge formation to diastolic myocardial stress/stiffness. Please note that for the initial experiments in membrane-permeabilized cardiomyocytes from human (Fig. 2) and rat (Fig. 3) the temperature was set at 15ºC. This relatively low temperature was used to avoid preparation deterioration and maintain the stability of the muscle preparation during several long-lasting series of activation. This was shown not to be problematic for the current experiments at 37ºC as a result of lower levels of activation in concert with a relatively brief protocol.

Four protocols were performed in which rat membrane-permeabilized cardiomyocytes were stretched from 1.8 to 2.4 μm sarcomere length. Conditions used are presented in Table 1. A representative recording is presented in Figure 4A showing diastolic force generation measured at 37ºC. Note that force is generated at 37ºC, which is not seen in the other three conditions. Comparison of 15ºC and 15ºC+Blebbistatin confirmed the previous observations that cross-bridge contribution to diastolic stiffness is nearly absent at non-physiological conditions, with a statistical difference found at a sarcomere length of 2.4 μm (Fig. 4B). However, comparison of 37ºC with 37ºC+Blebbistatin shows that the cross-bridge contribution is significantly higher, accounting for 76±1% of total diastolic stress averaged over all sarcomere lengths (Fig. 4B). Together these data support that in intracellular environments where high ADP and increased diastolic $[Ca^{2+}]$ are present, such as in the failing myocardium, the actomyosin

contribution to high diastolic stiffness may be substantially higher than previously considered and potentially represent a pathophysiological mechanism of HF with impaired diastolic function.

CK-inhibited rat cardiomyocytes show impaired relaxation and augmented contractility

To investigate the impact of inactivated CK activity and the consequent increase in cytosolic ADP levels, as to replicate the cytosolic environment of HF myocardium, intact rat cardiomyocytes were perfused with the irreversible CK antagonist iodoacetamide (15 μmoles/3 ml) during pacing at 1Hz and 37ºC. CK activity was inhibited by 38±7%. Contraction and Ca^{2+}-transient were simultaneously recorded in control and iodoacetamide-treated intact rat cardiomyocytes (Fig. 5). No significant alterations were observed between controls and iodoacetamide group at baseline (phase I) (Table 2, Supp Table 2 and Figs. 5A-5G). Control cardiomyocytes remained stable throughout the entire protocol for all parameters analyzed, i.e. no significant alterations were observed, (compared from phase I to phase III) with the exception for decreases of systolic and amplitude Ca^{2+}-signal, likely resulting from deterioration of the fluorescence signal over time (Table 2).

From phase I (baseline) to phase II (initial perfusion with iodoacetamide), diastolic and systolic sarcomere length were significantly reduced by 14±2% and 38±6%, respectively (Fig. 5B and Table 2), and sarcomere shortening increased by 24±5% (Fig. 5E and Table 2). In addition, cardiomyocyte shortening time was significantly increased without a significant change in shortening velocity (Table 2). The contractile changes were not mediated by changes in Ca^{2+}-transients, since there were no statistical alterations in diastolic Ca^{2+} (Fig. 5F) and the Ca^{2+}-relaxation time constant (tau) (Fig. 5G) from phase I to II.

Between phases II and III (after iodoacetamide perfusion) diastolic and systolic sarcomere lengths decreased significantly compared with baseline and phase II (Fig. 5B and Table 2). This was accompanied by 35±8% enhancement of sarcomere shortening compared with baseline (Fig. 5E). These latter contractile changes are associated with disturbed Ca^{2+}-handling, which includes significant increases of diastolic Ca^{2+} (20±6%,

Fig. 5F) with prolongation of Ca^{2+}-reuptake by 50±20% (tau) (Fig. 5G) compared with baseline (Table 2).

Together these data indicate that inhibiting CK, which elevates cytosolic ADP, in intact cardiomyocytes leads to impaired diastolic relaxation (i.e. limited diastolic re-lengthening). Initially, these alterations are independent of changes in the Ca^{2+} transient, but prolonged CK-inhibition was associated with diastolic Ca^{2+}-overload and slowing of Ca^{2+}-reuptake.

CK-inactivation decreases ventricular compliance in the presence of diastolic Ca^{2+}

Tian *et al.*(Tian *et al.*, 1997a; Tian *et al.*, 1997b) have previously shown that myocardial ADP elevation increases LVEDP and impairs myocardial relaxation in isolated rat hearts. However, the authors argued against a role of Ca^{2+} and solely attributed the latter effects to high [ADP] (Tian *et al.*, 1997a). To clarify whether ADP is the sole responsible factor for the reported increase in LVEDP, ventricular compliance was measured in isolated Langendorff-perfused rat hearts, in which CK was inactivated, in the absence and presence of the intracellular Ca^{2+}-chelator BAPTA-AM. Diastolic pressure-volume (PV) relationships were performed in hearts arrested in Ca^{2+}-free buffer following inhibition of CK with 2,4-dinitro-1-fluorobenzene (DNFB) and with BAPTA-AM (Fig. 6A). DNFB (20 μM) inhibited CK activity reaction by 42±8%, i.e. about equal to iodoacetamide effect. Diastolic PV slope increased in hearts perfused with DNFB in Ca^{2+}-free solution (Fig. 6B), indicative of reduced LV compliance. In other hearts, addition of BAPTA-AM (20μM) to Ca^{2+}-free solution (-Ca^{2+}+BATPA) did not change diastolic PV slope compared to -Ca^{2+} solution (Fig. 6B), but prevented the increase in diastolic PV slope following DNFB (Fig. 6B). To understand whether Ca^{2+} alone could account for the increased PV slope, 200 μM of caffeine was added to induce Ca^{2+}-release (Fig. 6B). The PV slope only showed a modest increase compared to CK-inhibition (Fig. 6B). Together, these results indicate that the combined presence of CK-inactivation and diastolic [Ca^{2+}] are needed for the observed decline in ventricular compliance in whole heart preparations.

Iodoacetamide and DNFB do not target myofilament proteins

To exclude direct effects of iodoacetamide or DNFB on myofilament proteins, membrane-permeabilized rat cardiomyocytes were measured before and after incubation with both CK-inhibitors. Maximal, submaximal Ca^{2+}-tension and F_{pas} were not changed in the presence of both inhibitors, indicative that both CK-inhibitors do not directly target myofilament protein functions (Table 3). No phosphorylation differences after iodoacetamide treatment in the intact rat cardiomyocytes (Figure 5) were found for the myofilament regulators of Ca^{2+}-sensitivity of force, including cardiac myosin-binding protein C, cardiac troponin I and myosin light chain, and the regulator of diastolic stiffness titin (data not shown). Altogether, these data strongly suggest that the observed CK-inhibition effects on diastolic impairment (Figs. 5 and 6) are due to its effects on myocardial energy reserve and independent of alterations of myofilament proteins.

Discussion

Diastolic function is both dependent on the rate of myocardial relaxation and passive properties of the wall that allow proper filling of the ventricle. In this study we provide evidence that synergistic actions of ADP and Ca^{2+} increase actomyosin interactions which elevate myocardial stiffness and limit proper filling of the heart. Our findings show that increased cross-bridge interactions may lead to diastolic dysfunction, in environments with elevated ADP and increased Ca^{2+}, evidenced by high cardiomyocyte stiffness and impaired diastolic re-lengthening, associated with limited ventricular compliance. In human and rat membrane-permeabilized cardiomyocytes, physiological levels of ADP led to an increased myofilament Ca^{2+}-sensitivity and stiffness, indicating a slowing or incomplete ventricular relaxation with high diastolic stiffness. In support, CK-inactivation limited restoration of diastolic length in intact rat cardiomyocytes and decreased ventricular compliance in whole rat hearts. Altogether these findings indicate that the increased [ADP] as observed in cardiac diseases, in which the metabolic function is compromised, significantly contributes to impaired diastolic function.

Elevated ADP and Ca^{2+} contribute to diastolic dysfunction

Combining studies in human and rat cardiomyocytes and hearts, we show that high ADP and increased Ca^{2+} enhance diastolic stress. In human and rat membrane-permeabilized cardiomyocytes a combination of Ca^{2+} and physiological ADP levels increased Ca^{2+}-sensitivity and cross-bridge stiffness (Figs. 2A and 2D, and Figs. 3A and 3C). Similar findings in the presence of supra-physiological levels of ADP have been shown to elevate Ca^{2+}-sensitivity and sarcomere stiffness in both skeletal rabbit myofibrils (Lu *et al.*, 2001; Tesi *et al.*, 2002; Siththanandan *et al.*, 2006) and rat trabeculae (Martyn *et al.*, 2007). Additionally, in intact rat cardiomyocytes CK-inhibition limited diastolic relaxation (Fig. 5B). LV compliance also declined in isolated rat hearts (Fig. 6). Similarly, it was previously shown by Tian *et al.*(Tian *et al.*, 1997a; Tian *et al.*, 1997b) that myocardial ADP accumulation increases LVEDP and impairs myocardial relaxation in isolated rat hearts. However, these authors argued that these changes are Ca^{2+}-independent (Tian *et al.*, 1997a). Our findings instead support the hypothesis that Ca^{2+} exacerbates the decline in LV

compliance in the presence of ADP. This is shown by the observation that buffering Ca^{2+} prevented the decrease in compliance during CK-inactivation (Fig. 6B). Conversely, a low concentration of caffeine (200 μM), to induce diastolic Ca^{2+}-release, showed a modest increase in PV slope compared to CK-inhibition (Fig. 6B). Thus, Ca^{2+} alone cannot completely account for the observed decline of LV compliance. These data suggest that in the absence of ADP resting diastolic Ca^{2+} has a relatively small contribution to diastolic stiffness and that ventricular compliance only declines in the presence of diastolic Ca^{2+} and high ADP caused by an active cross-bridge component. We provide evidence that high ADP and Ca^{2+}-dependent cross-bridge formation imposes high myocardial diastolic stiffness associated with greater than normal filling pressure, which contribute to diastolic dysfunction.

Augmented contractility following elevations of ADP and Ca^{2+}

On the basis of the observed diastolic impairment of intact rat cardiomyocytes we would expect a reduced fractional shortening, resulting from restricted recruitment of the Frank-Starling reserve. This would decrease the preload-induced force of contraction. In contrast, contractility was increased as evidenced by enhanced shortening capacity of intact rat cardiomyocytes following CK-inactivation (Fig. 5E). The greater than normal contractility can be explained by the increased Ca^{2+}-sensitivity of force production induced by physiological levels of ADP (Figs. 2A and 3A) and the Ca^{2+}-overload state promoted by CK-inactivation (Figs. 5F and 5G, and Table 2). Notably, our analysis reveals that the early inotropic response following CK-inactivation (phase II) is not caused by disturbed Ca^{2+}-transient (Figs. 5F and 5G, and Table 2) and thus explained by the ADP-induced increase of myofilament Ca^{2+}-sensitivity (Figs. 2A and Fig. 3A). However, prolonged CK-inhibition (phase III) still leads to increased contractility, but also significantly impairs diastolic re-lengthening (Fig. 5B), both of which are likely caused by systolic and diastolic Ca^{2+}-overload (Figs. 5F and 5G, and Table 2), respectively. Our results support the theory that CK-inactivation by increases of cytosolic ADP stimulates a Ca^{2+}-overload state. The Ca^{2+}-overload can result from: 1) the high Ca^{2+}-sensitivity promoted by ADP at the myofilaments; because the myofilaments are the main source for the buffering of Ca^{2+}

during the cardiac cycle (Bers, 2001), evidence from animal models supports that high myofilament Ca^{2+}-sensitivity increases cytosolic Ca^{2+}-binding with altered Ca^{2+}-homeostasis in animals (Baudenbacher *et al.*, 2008; Schober *et al.*, 2012); and/or 2) the high [ADP/ATP] ratio caused by ADP accumulation that reduces the free energy released from ATP hydrolysis (ΔG_{ATP}). Since the sarcoplasmic reticulum Ca^{2+}-ATPase, and other sarcolemmal pumps, including the Na^+/K^+-ATPase, operate close to their theoretical thermodynamic limits, the rise of [ADP] will favor diastolic Ca^{2+} rise (Allen & Orchard, 1987). However one cannot exclude that iodoacetamide also has an effect on Ca^{2+}-release via the ryanodine receptor as was shown in skeletal muscle from rabbits (Menshikova *et al.*, 2000). Hence, in our experiments 'artificial' elevation of Ca^{2+} that is not coupled to CK-inhibition may also be present.

Enhanced actomyosin interactions lead to high diastolic stress

It has been proposed that expression of the stiffer titin isoform or its altered phosphorylation represents a major contributor to high diastolic stiffness observed in HF patients with diastolic dysfunction (van Heerebeek *et al.*, 2012; Hamdani & Paulus, 2013). Within these studies, there is no evidence to support a role for altered cross-bridge interaction to high diastolic stiffness (van Heerebeek *et al.*, 2012; Hamdani & Paulus, 2013). In the majority of the studies membrane-permeabilized cardiomyocytes and muscle strips, with thick- and thin-filament extraction by KCl and KI, have been employed to study passive properties of HF myocardium (van Heerebeek *et al.*, 2012; Hamdani & Paulus, 2013). There are however limitations to the latter approaches that need to be addressed: 1) KCl and KI treatment is designed to assess the contribution of collagen to sarcomere stiffness as it removes thick- and thin-filament anchors in the sarcomere, and thereby abolishes any contribution of the actomyosin interaction (Granzier & Irving, 1995); 2) chemical digestion of the sarcolemma in membrane-permeabilized cardiomyocytes causes myofilament lattice expansion (and less approximation of myosin towards actin) that decreases force generation (McDonald & Moss, 1995; Konhilas *et al.*, 2002); 3) the relatively low temperature (15ºC) used in the experiments reduces cross-bridge active force, shortening velocity and power (Ranatunga & Coupland, 2010); 4) rapid shortening

and re-lengthening procedures mechanically detach myosin from actin (Huxley & Simmons, 1971; Brenner, 1991); and importantly 5) membrane-permeabilized cardiomyocytes lack important contributors in the intracellular milieu of normal and failing intact cardiomyocytes. These include the *in vivo* diastolic Ca^{2+} and cytosolic ADP. King *et al.*(King *et al.*, 2011) has recently reported that cross-bridges account for up to ~30% of diastolic stress at a sarcomere length of 2.1 μm using mouse membrane-permeabilized cardiomyocytes measured at 37ºC in the presence of dextran to normalize lattice spacing. In the present study we adapted this protocol to evaluate the cross-bridge contribution to diastolic stress in rat membrane-permeabilized cardiomyocytes under disease conditions, including those where metabolic function is affected that elevates ADP in the presence of diastolic Ca^{2+}, and showed that cross-bridges greatly contribute to diastolic stress (Fig. 4). Our data support the idea that cross-bridges may slow myocardial relaxation and impose high diastolic stiffness in disease, and in concert with titin and other diastolic mediators, contribute to diastolic dysfunction in HF patients. Importantly our results support the notion that the diastolic phase is not purely a passive process, but is accompanied by active stress.

Limitations

At present, we are unable to directly control intracellular ADP concentration in the intact cardiomyocytes and whole heart preparations. To increase cytosolic ADP, we opted for the use of two well known drugs that inhibit the CK reaction, i.e. iodoactemide and DNFB. Iodoacetamide allows us to estimate ADP elevations due to its well-characterized effects on the ATP-regeneration system (Tian *et al.*, 1997a). Based on previous findings using similar amounts of iodoactemide (Tian *et al.*, 1997a), ADP is estimated not to exceed 73 μM in our experiments, while ATP, PCr, inorganic phosphate and pH levels are unaltered. A limitation of iodoactemide which makes it unsuitable for whole heart experiments, is that it unspecifically targets enzymes of the glycolytic reaction (Tian *et al.*, 1997a). This is not problematic in single cells because of the short duration of the experiment (3 min). In contrast, this might be detrimental in the whole heart experiments, which required longer periods of CK-inhibition (30 min). Therefore, DNFB was used in those experiments, as it does not target enzymes of the glycolytic pathway (Westerblad & Lännergren, 1995).

However, using DNFB we are unable, to our knowledge, to carefully estimate the expected intracellular ADP concentration. We believe that overall the effects of both drugs are interchangeable between experiments, which is partly supported by similar inhibition of the CK reaction (~40%).

Conclusion

Based on our experimental data, we propose that conditions which elevate myocardial ADP and Ca^{2+} contribute to diastolic dysfunction by increasing residual actomyosin interactions. This leads to high cardiomyocyte stiffness and impaired relaxation, associated with limited ventricular compliance. Our study provides a mechanistic basis for how the elevated ADP and diastolic Ca^{2+}-overload can contribute to diastolic dysfunction in HF patients.

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Additional information

Competing interests

None.

Authors contributions

The objective of our study was to demonstrate that elevations of ADP in the presence of low cytosolic Ca^{2+}, at concentrations present during the diastolic phase *in vivo*, are sufficient to cause diastolic abnormalities. All authors approved the final version of the manuscript. Conception and design of the experiments: V.S. devised, designed and performed the project, analyzed data and wrote the manuscript; A.N. performed experiments, analyzed data and revised the manuscript; M.M. performed experiments, analyzed data and revised the manuscript; E.D.F. performed experiments, analyzed data and revised the manuscript; I.A.E.B. performed experiments, analyzed data and revised the manuscript; R.C.I.W. wrote and revised the manuscript; C.d.R. collected the human cardiac samples and revised manuscript; M.H. analyzed data and revised the manuscript; E.W. wrote and revised the manuscript; G.J.M.S. supervised the project and revised the manuscript; J.T. wrote and revised the manuscript; D.W.D.K. supervised the project, wrote and revised the manuscript; J.v.d.V. supervised the project, wrote and revised the manuscript.

Translational Perspective

Here we show that actomyosin-related force development may be an important contributor to high diastolic stiffness and may explain the development of diastolic dysfunction in environments where high ADP and increased diastolic $[Ca^{2+}]$ are present, such as in the failing myocardium. These findings establish a clear link between deficits of myocardial energy reserve and disturbed Ca^{2+}-handling, which together leads to inappropriate cross-bridge formation and high diastolic stiffness. These findings may provide insight in how energy-sparing therapies (that reduce cellular ATP usage), such as beta-blockers, angiotensin-receptor blockers and angiotensin converting enzyme inhibitors, improve symptoms and reduce mortality in congestive HF patients (McMurray & Pfeffer, 2005). Our data highlight the need for more targeted therapies aimed at lowering myocardial ADP levels to improve the prognosis of human HF.

Table 1. Solution's composition of the 4 conditions tested.

Condition	[Ca^{2+}] (µM)	Dextran	Blebbistatin (µM)	PCr (mM)	MgATP (mM)	MgADP (µM)
Unphysiological						
15ºC	<0.001	-	-	14.5	10	-
15ºC+Blebbistatin	<0.001	-	30	14.5	10	-
Physiological						
37ºC	0.15	4%	-	-	10	100
37ºC+Blebbistatin	0.15	4%	30	-	10	100

PCr, Phosphocreatine; pH was adjusted to 7.1 at 15ºC and at 37ºC with KOH and ionic strength was adjusted to 180 mM with KCl; 2 mM free Mg^{2+}, 10 mM MgATP, 7 mM EGTA and 100 mM BES were present in all solutions; Experiments performed at 15ºC were used to mimic conditions currently employed to study diastolic stiffness, while experiments at 37ºC better recapitulate the *in vivo* diseased heart.

Table 2. Cardiomyocyte shortening and Ca^{2+}-transient in rat cardiomyocytes with and without iodoacetamide.

	Phase I		Phase II		Phase III	
	Ctrl-group	**IA-group**	**Ctrl-group**	**IA-group**	**Ctrl-group**	**IA-group**
Shortening						
Diastolic SL (µm)	1.85±0.01	1.85±0.01	1.85±0.01	1.83±0.01[§]	1.85±0.01	1.81±0.01[‡#]
Shortening velocity (µm/s)	-4.9±0.3	-4.5±0.4	-5.0±0.2	-5.1±0.3	-5.2±0.3	-5.3±0.3[‡]
Time to peak shortening (s)	0.060±0.004	0.060±0.003	0.061±0.004	0.065±0.003[§]	0.061±0.004	0.066±0.003[‡]
Sarcomere shortening (%)	8.2±0.5	7.3±0.5	8.3±0.4	9.0±0.6[§]	8.7±0.4	9.4±0.6[‡]
Systolic SL (µm)	1.70±0.01	1.71±0.02	1.70±0.01	1.66±0.02[§]	1.69±0.01	1.63±0.01[‡#]
Relaxation velocity (µm/s)	3.5±0.2	3.2±0.3	3.6±0.2	3.5±0.3	3.7±0.2	3.6±0.3
Time to 90% relaxation (s)	0.15±0.01	0.17±0.01	0.15±0.01	0.17±0.01	0.15±0.01	0.21±0.03
Ca^{2+}-transient						
Diastolic Ca^{2+} (F340/380)	1.00±0.09	0.98±0.08	0.99±0.09	1.05±0.08	0.96±0.08	1.17±0.08[‡]
Time to peak Ca^{2+} release (s)	0.028±0.002	0.028±0.001	0.028±0.001	0.031±0.002	0.030±0.001	0.036±0.003[‡]
Systolic Ca^{2+} (F340/380)	1.58±0.15	1.57±0.14	1.51±0.15[§]	1.54±0.15	1.39±0.12[‡]	1.56±0.12
Ca^{2+} amplitude (F340/380)	0.58±0.08	0.60±0.07	0.49±0.07[§]	0.49±0.07[§]	0.42±0.06[‡#]	0.38±0.06[‡#]
Tau (s)	0.095±0.005	0.094±0.006	0.094±0.005	0.096±0.007	0.096±0.007	0.132±0.015[‡#]

Ctrl, Control; IA, Iodoacetamide; SL, sarcomere length. Data was compared using repeated measures 1-way ANOVA followed by Bonferroni *post hoc* test. $p < 0.05$ was considered significant; [§]1-way ANOVA *post hoc* test phase I versus corresponding phase II; [‡]1-way ANOVA *post hoc* test phase I versus corresponding phase III; [#]1-way ANOVA *post hoc* test phase II versus corresponding phase III; Detailed statistics in Table S2. 7 heart samples from adult wild-type male Wistar rats were used; 17 to 18 (Ctrl-group) and 17 to 20 (IA-group) cardiomyocytes were analysed.

Table 3. Response of Ca^{2+}-stimulated maximal and submaximal contraction without and with iodoacetamide or DNFB, in left ventricular rat membrane-permeabilized cardiomyocytes at sarcomere length 2.2 μm.

Sample	before incubation	after incubation	N/n
Iodoacetamide (5 mM)			N=3, n=11
32 μM Ca^{2+}-F_{act} (kN/m^2)	22.3±2.7	21.0±2.3	
4.0 μM Ca^{2+}-F_{act} (kN/m^2)	17.1±2.0	16.2±1.8	
F_{pas} (kN/m^2)	1.7±0.2	1.6±0.2	
DNFB (20 μM)			N=3, n=9
32 μM Ca^{2+}-F_{act} (kN/m^2)	20.0±1.5	19.6±1.5	
4.0 μM Ca^{2+}-F_{act} (kN/m^2)	14.0±1.5	13.8±1.5	
F_{pas} (kN/m^2)	1.2±0.2	1.1±0.2	

N, number of rats; n, number of cardiomyocytes. Cardiomyocytes were measured before and after incubation with 5 mM iodoacetamide or 20 μM DNFB, in relaxing solution, for 5 or 30 minutes, respectively. Data was compared using a paired-sampled Student's t-Test. p<0.05 was considered significant.

Fig. 1. ADP causes force production in human left ventricular membrane-permeabilized cardiomyocytes. *A,* Representative tension recordings of a single cardiomyocyte isolated from a end-stage failing human heart at 2.2 μm sarcomere length during maximal Ca^{2+} (32 μM) and ADP-activation (10 mM) at 15ºC and 1 mM ATP. *B,* Normalized force-ADP relationship of human cardiomyocytes. 9 to 11 cardiomyocytes from 3 failing human hearts were used.

Fig. 2. Ca^{2+}-sensitivity and cross-bridge stiffness increases in the presence of ADP in human left ventricular membrane-permeabilized cardiomyocytes. *A,* Ca^{2+}-force relations were constructed without and with [ADP] at 15ºC and 1 mM ATP. Light blue panel depicts the free $[Ca^{2+}]$ range in *in vivo* cardiomyocytes (i.e. [~0.15-1.6 μM]). Data was compared using 1-way ANOVA followed by Bonferroni *post hoc* test. All data show significant differences compared with 'control (no [ADP])'. *B,* Normalization of ktr as a function of Ca^{2+}-activated isometric force. The ktr-force relationship shifted to the right and downwards with [ADP] (R^2=0.93, R^2=0.88, R^2=0.76, R^2=0.65, R^2=0.55 and R^2=0.81, from 0-6 mM ADP respectively). A linear mixed model assuming force as a continuous variable indicates significant differences for the interactions of 2 and 6 mM ADP compared with 'control (no [ADP])'. *C,* Residual force recording tracings. Top: Residual force recordings after a slack-restretch test at high Ca^{2+} (32 μM) without or with increasing [ADP]. Recording of 2 mM ADP is omitted from the graph due to overlap with 6 mM ADP recording. Bottom: Length changes of piezoelectric motor by 30% of cardiomyocyte's original length is depicted. Residual force was calculated from the initial force recovery reached after the length change (dashed lines) and normalized to each total steady-state force reached before length change. Measurements are normalized to each corresponding total force. *D,* Significant increases in residual force enhancement at high Ca^{2+} without or with various [ADP] are observed after a slack-restretch test, indicative for increased sarcomere stiffness. Values are normalized to each corresponding total relative force reached before the slack-restretch procedure. Data was compared using 1-way ANOVA followed by Bonferroni *post hoc* test. [*]p<0.05 versus 'control (no [ADP])'.

9 to 11 cardiomyocytes from 3 end-stage human failing hearts were used for each ADP concentration.

Fig. 3. Ca^{2+}-sensitivity and cross-bridge stiffness increases in the presence of ADP in left ventricular rat membrane-permeabilized cardiomyocytes. *A,* Ca^{2+}-force relations were constructed without and with 0.1 mM ADP at sarcomere lengths of 2.2 μm and at 15ºC and 1 mM ATP. Data was compared using paired t-Test. Ca^{2+}-sensitivity of force significantly increases in the presence of 0.1 mM ADP compared with 'control (no [ADP])'. *B,* Normalization of ktr as a function of Ca^{2+}-activated isometric force. The ktr-force relationship shifted to the right and downwards with 0.1 mM ADP (R^2=0.79 and R^2=0.95, 'control (no [ADP])' and 0.1 mM ADP, respectively). *C,* Significant increases in residual force enhancement at high Ca^{2+} with 0.1 mM ADP are observed after a slack-restretch test compared with 'control (no [ADP])', indicative for increased sarcomere stiffness. Values are normalized to each corresponding total relative force reached before the slack-restretch procedure. Data was compared using paired t-Test. *p<0.05 versus 'control (no [ADP])'. 12 cardiomyocytes from 4 adult wild-type male Wistar rat hearts were used.
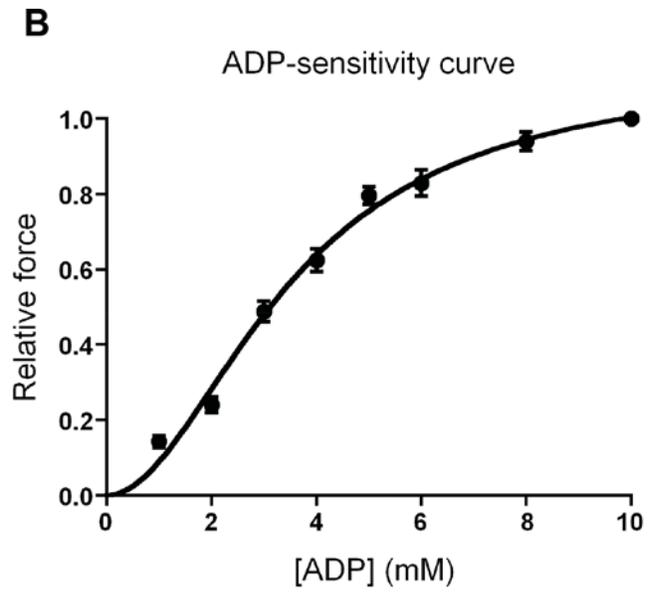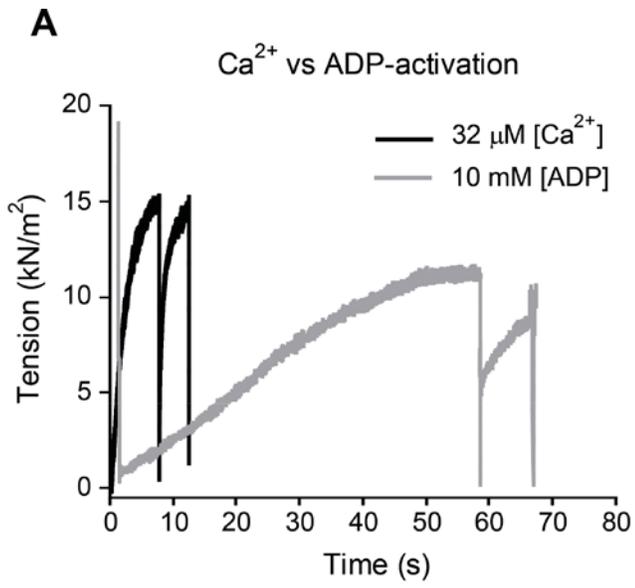
Fig. 4. Re-evaluation of the influence of cross-bridges on *in vitro* diastolic stress with *in vivo* conditions. *A,* Representative force recording example of a left ventricular single rat cardiomyocyte moved from a non-activating solution at 37ºC with 14.5 mM PCr and <0.001 μM Ca^{2+} to an activating solution at 37ºC in the presence of diastolic Ca^{2+} (0.15 μM), dextran (4%), 100 μM ADP and 10 mM ATP. Upper tracing: solutions used in the protocol are depicted. Middle: Length changes of piezoelectric motor is depicted. For measurement of passive force, a slack-restretch procedure of 30% of cardiomyocyte's original length is performed. Bottom: Force recording after a slack-restretch test is depicted. Diastolic tension value was taken from the 30% slack procedure over a period of 10 seconds (period from 30 to 40 seconds). *B,* Tension-sarcomere length relations are depicted from left ventricular rat membrane-permeabilized cardiomyocytes. Rat cardiomyocytes were stretched from 1.8 to 2.4 μm and diastolic stress was measured in the following conditions: 37ºC (Dextran (4%), diastolic Ca^{2+} (0.15 μM) and 100 μM
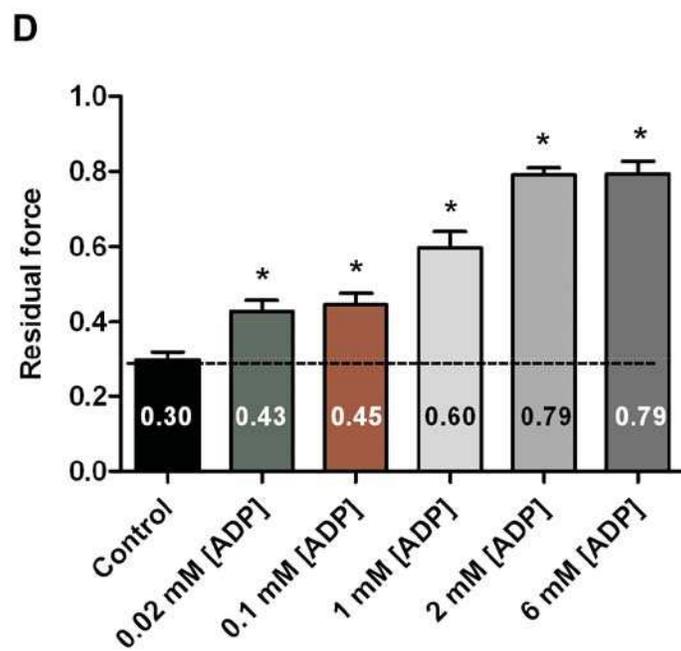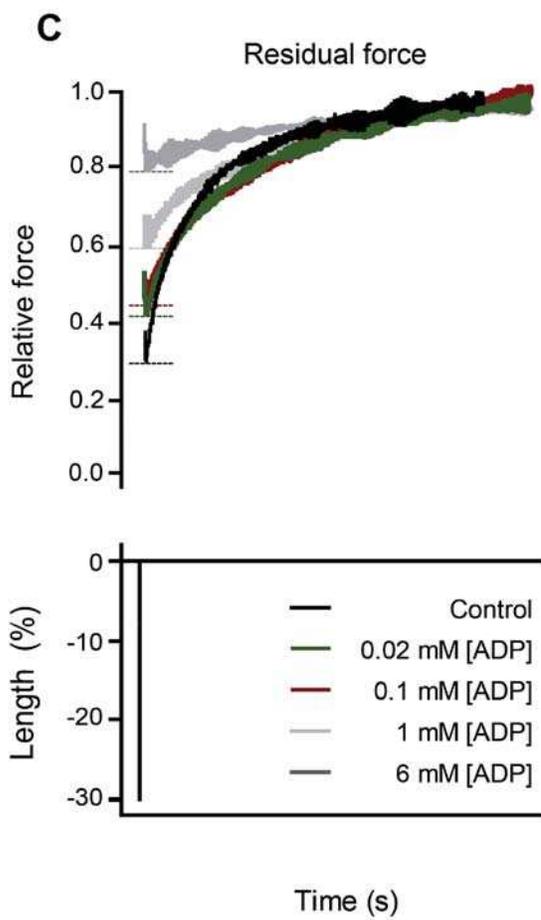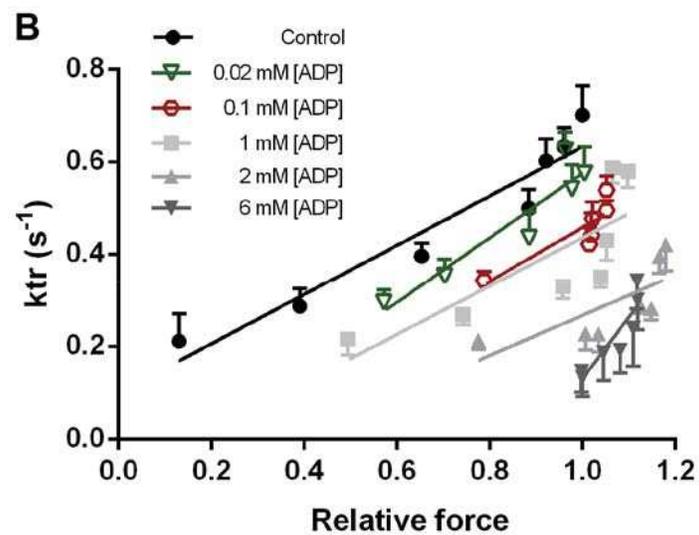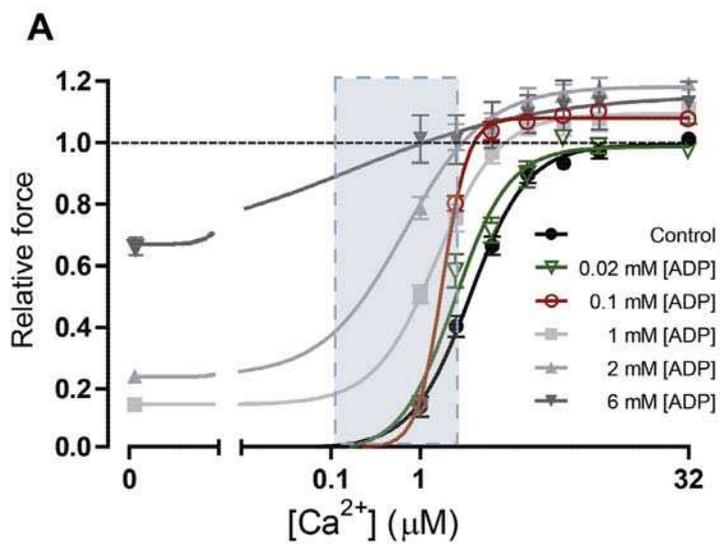
ADP) in the absence (dark exponential curve) and presence of Blebbistatin ($37^{\circ}C$ + Blebbistatin; dashed dark exponential curve); and $15^{\circ}C$ (with 14.5 mM PCr and <0.001 μM Ca^{2+}) in the absence (gray exponential curve) and presence of Blebbistatin ($15^{\circ}C$ + Blebbistatin; dashed gray exponential curve). All solutions contained 10 mM ATP. Comparison of $37^{\circ}C$ with $37^{\circ}C$+Blebbistatin shows that the cross-bridge contribution accounts for 76±1% of total diastolic stress averaged over all sarcomere lengths. Data was compared using unpaired t-Test within independent groups measured at similar temperature for each individual sarcomere length. [*]$p<0.05$ versus 'in the presence of Blebbistatin'. 12 to 16 cardiomyocytes from 5 adult wild-type male Wistar rat hearts were used.
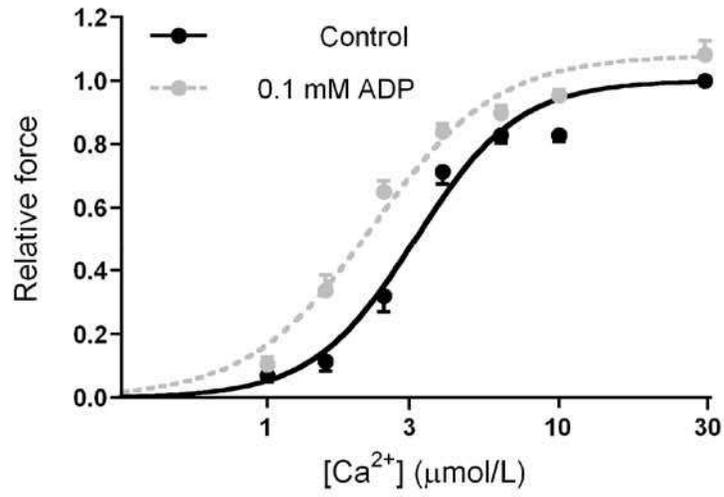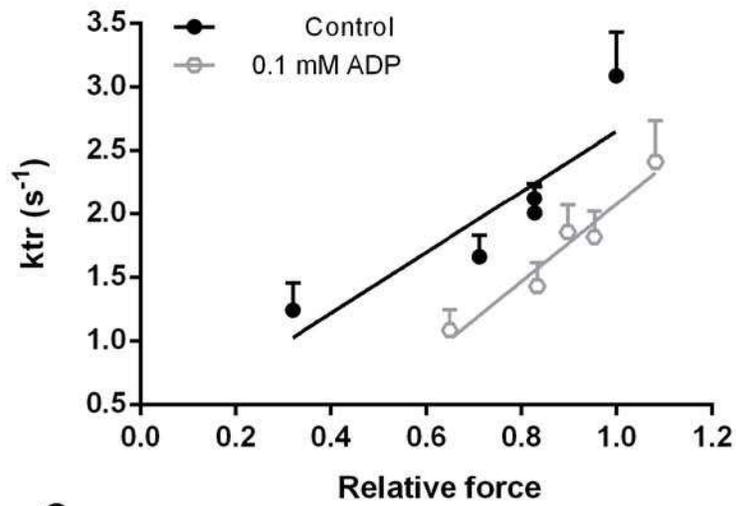
Fig. 5. Creatine kinase-inhibited cells show decreased diastolic length but augmented contractility in intact left ventricular rat cardiomyocytes. Example of sarcomere length and Ca^{2+}-transient recordings of left ventricular single unloaded intact rat cardiomyocytes with iodoacetamide treatment at 1Hz pacing. *A,* Iodoacetamide treatment. Phase (**I**). Baseline. Measurements just prior to iodoacetamide administration (cardiomyocytes paced for 3-5 minutes). Iodoacetamide (**start-stop**). Perfusion (at 1 ml/min) was performed for 3 min with 5 mM iodoacetamide resulting in a total dose of 15 μmoles/3 ml. Phase (**II**). Initial perfusion with iodoacetamide. Cardiomyocytes were allowed 1 min to adjust to iodoacetamide perfusion and measurements were recorded for 4 min. Phase (**III**). Post-iodoacetamide perfusion. Measurements were carried out ranging from 3-10 min depending on cardiomyocytes survival. Phase III was typified by diastolic $[Ca^{2+}]$ increases as well as systolic and diastolic sarcomere length alterations. **Note**: example of cell death in the last 100 seconds of recording. *B,* Alterations of diastolic and systolic sarcomere length from phase I (baseline), throughout phase II (initial perfusion with iodoacetamide) to phase III (after iodoacetamide perfusion), in iodoacetamide (IA)-treated cells. Relative sarcomere length changes compared with phase I of each corresponding group. [*]versus Phase I. *C,* Control recording protocol (no treatment) tracing. Similar to (**A**) except, instead of iodoacetamide treatment, cardiomyocytes were perfused with buffer solution. **Note**: during both protocols cardiomyocytes were continuously perfused with buffer solution (at 1 ml/min) with the

exception being the 3 minute period of iodoacetamide perfusion in (**A**). ***D,*** Alterations of diastolic and systolic sarcomere length from phase I (baseline), throughout phase II (similar perfusion time as phase II in (**B**)) to phase III (similar perfusion time as phase III in (**B**)), in control cells. Relative sarcomere length changes compared with phase I of each corresponding group. ***E,*** % increase change of sarcomere shortening. ***F,*** % increase of diastolic Ca^{2+}-level. ***G,*** % delay of the Ca^{2+}-relaxation time constant, tau, indicative of Ca^{2+}-reuptake. Figures 5E, 5F and 5G are all % changes relative to phase I in IA-treated cells. Corresponding data are presented in Table 2. [*]p<0.05 versus Phase I. Data was compared using 1-way ANOVA followed by Bonferroni *post hoc* test. 7 heart samples from adult wild-type male Wistar rats were used; 17 to 18 (Ctrl-group) and 17 to 20 (IA-group) cardiomyocytes were analysed.

Fig. 6. Left ventricle (LV) compliance declines following creatine kinase (CK)-inactivation in isolated rat hearts. ***A,*** Mean diastolic pressure-volume (PV) relationship in Langendorff-perfused hearts arrested in Ca^{2+}-free buffer without and with inhibition of CK. Hearts (N=6) were perfused with nominally Ca^{2+}-free solution for 60 min then the diastolic PV relationship was measured ($-Ca^{2+}$). 2,4-dinitro-1-fluorobenzene (DNFB; 20 μM) was then perfused for a further 30 min and measurements repeated ($-Ca^{2+}$+DNFB). In a separate group of hearts (N=5), the intracellular Ca^{2+}-chelator BAPTA-AM (20μM) was added to $-Ca^{2+}$ solution and perfused for 60 min ($-Ca^{2+}$+BAPTA), followed by perfusion for 30 min with DNFB ($-Ca^{2+}$+DNFB+BAPTA). ***B,*** Slope of the diastolic PV relationship. DNFB reduced left ventricle compliance in $-Ca^{2+}$ solution, shown by a steeper diastolic PV relationship. BAPTA did not change diastolic PV slope compared to $-Ca^{2+}$. However, BAPTA prevented the increase in diastolic PV slope following DNFB perfusion. In a separate group of hearts (N=2), addition of low concentration of caffeine (200 μM) in Ca^{2+}-free solution appears to be sufficient to cause diastolic Ca^{2+}-leak, indicative from the increase in diastolic PV slope. Although to a lesser extent than $-Ca^{2+}$+DNFB. Data was compared using 2-way repeated measures ANOVA followed by Bonferroni *post hoc* test. [*]p<0.05.

A Ca²⁺ vs ADP-activation

B ADP-sensitivity curve

A

Relative force vs [Ca²⁺] (μM)

Legend:
- Control (●)
- 0.02 mM [ADP] (▽)
- 0.1 mM [ADP] (○)
- 1 mM [ADP] (■)
- 2 mM [ADP] (▲)
- 6 mM [ADP] (▼)

B

k_{tr} (s^{-1}) vs Relative force

Legend:
- Control (●)
- 0.02 mM [ADP] (▽)
- 0.1 mM [ADP] (○)
- 1 mM [ADP] (■)
- 2 mM [ADP] (▲)
- 6 mM [ADP] (▼)

C

Residual force

Relative force vs Time (s)

Length (%) vs Time (s)

Legend:
- Control (black)
- 0.02 mM [ADP] (green)
- 0.1 mM [ADP] (red)
- 1 mM [ADP] (light gray)
- 6 mM [ADP] (dark gray)

D

Residual force

| Control | 0.02 mM [ADP] | 0.1 mM [ADP] | 1 mM [ADP] | 2 mM [ADP] | 6 mM [ADP] |
| 0.30 | 0.43 * | 0.45 * | 0.60 * | 0.79 * | 0.79 * |

A

B

C

A

$[Ca^{2+}]$
0.001 μM

37°C

0.15 μM $[Ca^{2+}]$ + 100 μM [ADP]

with PCr
no Dextran

no PCr
Dextran (4%)

B

Diastolic cross-bridge contribution

- 37°C
- 37°C + Blebbistatin
- 15°C
- 15°C + Blebbistatin

76±1%

A

B

Passive pressure-volume relation