JCI The Journal of Clinical Investigation

Role of MgADP in the development of diastolic dysfunction in the intact beating rat heart.

R Tian, … , S A Camacho, J S Ingwall

J Clin Invest. 1997;99(4):745-751. https://doi.org/10.1172/JCI119220.

Research Article

Sarcomere relaxation depends on dissociation of actin and myosin, which is regulated by a number of factors, including intracellular [MgATP] as well as MgATP hydrolysis products [MgADP] and inorganic phosphate [Pi], pHi, and cytosolic calcium concentration ([Ca2+]c). To distinguish the contribution of MgADP from the other regulators in the development of diastolic dysfunction, we used a strategy to increase free [MgADP] without changing [MgATP], [Pi], or pHi. This was achieved by applying a low dose of iodoacetamide to selectively inhibit the creatine kinase activity in isolated perfused rat hearts. [MgATP], [MgADP], [Pi], and [H+] were determined using 31P NMR spectroscopy. The [Ca2+]c and the glycolytic rate were also measured. We observed an approximately threefold increase in left ventricular end diastolic pressure (LVEDP) and 38% increase in the time constant of pressure decay ($P < 0.05$) in these hearts, indicating a significant impairment of diastolic function. The increase in LVEDP was closely related to the increase in free [MgADP]. Rate of glycolysis was not changed, and [Ca2+]c increased by 16%, which cannot explain the severity of diastolic dysfunction. Thus, our data indicate that MgADP contributes significantly to diastolic dysfunction, possibly by slowing the rate of cross-bridge cycling.

Role of MgADP in the Development of Diastolic Dysfunction in the Intact Beating Rat Heart

Rong Tian,* Michael E. Christe,* Matthias Spindler,* James C.A. Hopkins,* Jessica M. Halow,‡ S. Albert Camacho,§ and Joanne S. Ingwall*

**NMR Laboratory for Physiological Chemistry, Cardiovascular Division, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts 02115; ‡Cardiovascular Research Institute, San Francisco General Hospital, University of California, San Francisco, California 94121; and §Department of Medicine (Cardiology), San Francisco General Hospital, University of California, San Francisco, California 94121*

Abstract

Sarcomere relaxation depends on dissociation of actin and myosin, which is regulated by a number of factors, including intracellular [MgATP] as well as MgATP hydrolysis products [MgADP] and inorganic phosphate [Pi], pH_i, and cytosolic calcium concentration ($[Ca^{2+}]_c$). To distinguish the contribution of MgADP from the other regulators in the development of diastolic dysfunction, we used a strategy to increase free [MgADP] without changing [MgATP], [Pi], or pH_i. This was achieved by applying a low dose of iodoacetamide to selectively inhibit the creatine kinase activity in isolated perfused rat hearts. [MgATP], [MgADP], [Pi], and $[H^+]$ were determined using ^{31}P NMR spectroscopy. The $[Ca^{2+}]_c$ and the glycolytic rate were also measured. We observed an approximately threefold increase in left ventricular end diastolic pressure (LVEDP) and 38% increase in the time constant of pressure decay ($P < 0.05$) in these hearts, indicating a significant impairment of diastolic function. The increase in LVEDP was closely related to the increase in free [MgADP]. Rate of glycolysis was not changed, and $[Ca^{2+}]_c$ increased by 16%, which cannot explain the severity of diastolic dysfunction. Thus, our data indicate that MgADP contributes significantly to diastolic dysfunction, possibly by slowing the rate of cross-bridge cycling. (*J. Clin. Invest.* 1997. 99:745–751.) Key words: cross-bridge cycling • calcium • ATP • inorganic phosphate • relaxation

Introduction

Muscle contraction and relaxation are determined by the cyclic interaction between myosin and actin. This interaction, known as cross-bridge cycling, consists of the attachment of the two proteins, a change in their conformation, and consequently the power stroke and detachment. The chemical energy used to support cross-bridge cycling is produced during MgATP hydrolysis by myosin ATPase. Studies of these proteins in solution have defined the key steps in this cyclic interaction (1, 2). In the absence of nucleotides, actin and myosin form a strong complex. The complex dissociates upon the binding of MgATP to myosin. Myosin hydrolyzes MgATP and the products of hydrolysis, inorganic phosphate (Pi)[1] and MgADP, are released sequentially. Pi is released when the actomyosin complex undergoes a conformational change which leads to force generation (3). MgADP must then be released before MgATP binds, enabling the actin and myosin to enter a second cycle. Thus, any alterations at the levels of MgATP binding, myosin ATPase activity, or the subsequent release of either MgADP or Pi may affect cross-bridge cycling, resulting in abnormal contraction and relaxation. Previous studies using skinned skeletal muscle fibers showed that an increase in free [Pi] inhibits the conformational change of the actomyosin complex and thereby influences the power stroke (4, 5). Using similar preparations, it was also shown that an increase in free [MgADP] reduced fiber velocity, possibly due to its interference with MgATP binding to myosin (6).

The regulation of cross-bridge cycling has so far only been studied using isolated contractile proteins or skinned fibers in which only a single contraction is elicited. In most of these experiments, [MgADP] was in mM range, which is one to two orders of magnitude higher than typical physiological levels. Regulation of the rhythmic contraction at the whole organ level, and, in particular, the role of MgADP in systolic and diastolic function of an intact heart, is still lacking. We reasoned that increased free [MgADP] in an intact cell, at a concentration achievable physiologically, could decrease the rate of MgADP detachment and thus slow down the rate of cross-bridge cycling, leading to an impairment of relaxation at the whole organ level.

Here we propose a strategy for testing whether this is the case for an intact beating heart: by applying a low dose of iodoacetamide (IA), a sulfhydryl group modifier, we were able to irreversibly inhibit creatine kinase (CK) without affecting other major MgATP synthesis and utilization pathways in an isolated buffer-perfused heart (7, 8). The selective inhibition of the CK reaction blocked the primary mechanism that keeps a low free [MgADP] in the cell. Since the MgATP synthesis via oxidative phosphorylation is unaffected, this approach offers an unique method to alter free [MgADP] without changing either [MgATP] or [Pi] in a beating heart at baseline workload.

Address correspondence to Rong Tian, M.D., Ph.D., NMR Laboratory for Physiological Chemistry, Brigham and Women's Hospital, 221 Longwood Avenue, Room 247, Boston, MA 02115. Phone: 617-732-6994; FAX: 617-732-6990; E-mail: rong@bustoff.bwh.harvard.edu

Received for publication 17 June 1996 and accepted in revised form 3 December 1996.

J. Clin. Invest.

0021-9738/97/02/0745/07 $2.00
Volume 99, Number 4, February 1997, 745–751

1. *Abbreviations used in this paper:* $[Ca^{2+}]_c$, cytosolic calcium concentration; CK, creatine kinase; IA, iodoacetamide; LVEDP, left ventricular end diastolic pressure; Pi, inorganic phosphate; τ_{Ca}, time constant for the monoexponential decline of the cytosolic calcium transient; τ_p, time constant for the monoexponential decline of left ventricular pressure.

Using this approach, we tested the hypothesis that an increase in free [MgADP] may cause an increase in left ventricular end diastolic pressure (LVEDP), a measurement of myocardial relaxation in an isolated, isovolumic rat heart. To take into consideration the other regulators of cross-bridge cycling, we also measured myocardial high energy phosphate content and intracellular pH (pH_i) by ^{31}P NMR spectroscopy. The trigger of the cross-bridge cycling, cytosolic calcium concentration ($[Ca^{2+}]_c$), was determined by indo-1 fluorescence measurement. Furthermore, recent evidence suggests that MgATP derived from glycolysis may play an important role in the maintenance of LVEDP (9). Because IA treatment has been shown to inhibit glycolytic enzyme activities, we also directly measured myocardial glycolytic flux in the absence and presence of IA.

We found a dose-dependent relationship between increases in LVEDP and free [MgADP] in IA-treated hearts. There was only a modest increase in $[Ca^{2+}]_c$ and there were no changes in [MgATP], [Pi], pH_i, or glycolytic rate. These results suggest that inhibition of the cross-bridge dissociation rate by increased [MgADP] is a likely contributor to diastolic dysfunction at a constant and high [MgATP]. Furthermore, these effects are independent of [Pi] and $[H^+]$.

Methods

Isolated perfused heart. Male Sprague-Dawley rats, weighing 375–425 g, were anesthetized by injection of 30 mg i.p. of sodium pentobarbital. Excised hearts were perfused by Langendorff mode. Briefly, aortic retrograde perfusion was maintained at a constant perfusion pressure of 100 mmHg at 37°C. Adequate coronary perfusion was obtained by incision of the root of the pulmonary artery. Thebesian vein drainage was facilitated by an apical vent. A water–filled latex balloon was inserted into the left ventricle through an incision in the left atrium for constant monitoring of left ventricular pressure and heart rate. Coronary flow was measured by collection of coronary effluent from the suction tube. The perfusate contained (mM): NaCl, 118.0; KCl, 4.7; $CaCl_2$, 1.75; $MgSO_4$, 1.2; EDTA, 0.5; $NaHCO_3$, 25.0; and glucose, 11.0 (pH 7.4 when gassed with 95% O_2, 5% CO_2 at 37°C). All hearts were allowed to equilibrate for 15–20 min when LVEDP was set at 5–10 mmHg by inflating the balloon in the left ventricle.

Four protocols were performed for this study. In protocol I, increasing doses of IA (total dose of 0, 30, 90 or 120 μmol, dissolved in H_2O, obtained from Sigma Chemical Co., St. Louis, MO) were infused into separate groups of hearts over a period of 15–30 min at a speed of 0.25 ml/min. Left ventricular function and ^{31}P NMR spectra defining the effect of IA treatment were collected before and after the infusion period. In protocol II, the temporal relationship between free [MgADP] and LVEDP was determined. Hearts were infused with either vehicle (H_2O) or IA for 30 min and estimates of cardiac function and ^{31}P NMR spectra were obtained at 4-min intervals. In protocol III, left ventricular pressure and indo-1 fluorescence signal were simultaneously collected immediately before and after infusion of vehicle or IA. EDTA was excluded and $[Ca^{2+}]$ was adjusted to 1.25 mM in the perfusate used for this protocol. Hearts were paced at 5 Hz, and left ventricular pressure was measured using a solid state, high fidelity transducer (Millar Instruments Inc., Houston, TX) attached to the water–filled latex balloon. Under these conditions, LVEDP for hearts supplied with 90 μmol of IA was 20 mmHg, a value similar to LVEDP for unpaced, non-dye-loaded hearts supplied with 120 μmol of IA. In protocol IV, glycolytic rate was measured in hearts treated either with vehicle (H_2O) or IA. At the end of the experiment, all hearts were freeze clamped with aluminum tongs cooled to the temperature of liquid nitrogen and stored at −80°C for biochemical assays.

^{31}P NMR spectroscopy. ^{31}P NMR spectra were obtained at 161.94 MHz on a GE-400 Omega spectrometer (GENMR Instruments, Fremont, CA). The heart was placed in an NMR sample tube and inserted into a $^1H/^{31}P$ double tuned probe which was situated in a 20-mm bore, 9.4 T superconducting magnet. Spectra were collected without proton decoupling at a pulse width of 27 μs, pulse angle of 60°, recycle time of 2.14 s and a sweep width of 6,000 Hz. Spectra were obtained by averaging the signals from 104 free induction decays during a 4-min period. Spectra were analyzed using 20 Hz exponential multiplication and zero and first order phase corrections. Each resonance peak was fitted to a Lorentzian function and the area under each peak was calculated by a commercially available program NMR1 (NMRi, Syracuse, NY). By comparing the peak areas of fully relaxed (recycle time 10 s) and those of partially saturated (recycle time 2.14 s) spectra, the correction factors for saturation were calculated for [β-P] MgATP (1.0), phosphocreatine (PCr, 1.2), and Pi (1.15).

In pilot studies, [MgATP] and total creatine [Cr] have been determined biochemically in the isolated perfused heart preparation at the end of the 20-min stabilization period. Using a value of 0.155 mg protein/mg blotted wet tissue and the reported value of 0.48 ml intracellular water/mg blotted wet tissue (10), [MgATP] and total [Cr] were calculated to be 11.7 mM and 27.4 mM respectively in these hearts (8). This value of [MgATP] was used to calibrate the [β-P] MgATP peak area of the NMR spectrum obtained during the initial equilibration period. The [PCr] and [Pi] were calculated by multiplying the ratio of their peak areas to that of [β-P] MgATP by 11.7 mM. The changes in [MgATP], [PCr], and [Pi] during the protocol were assessed as the percentage changes of their corresponding peak areas from the initial baseline spectrum. pH_i was determined by comparing the chemical shift of Pi and PCr in each spectrum to values from a standard curve.

Cytosolic free [MgADP] was calculated using the equilibrium constant of CK reaction (11) and values obtained by NMR spectroscopy and biochemical assay: $[MgADP] = ([MgATP][\text{free Cr}])/([PCr][H^+]K_{eq})$, where $K_{eq} = 1.66 \times 10^9\ M^{-1}$. When the activity of an enzyme is inhibited, the rate constants for the forward and reverse reactions are both decreased, and the K_{eq} is not altered. Thus, the equilibrium of the CK reaction is maintained even for the group of hearts with only 2% enzyme activity (0.25 IU/mg protein at 37°C). The remaining CK activity is greater than the ATP synthesis rate estimated by measuring myocardial oxygen consumption (0.16 μmol ATP/min per mg protein) (12). This prediction is further supported by our previous experimental observation that [PCr] decreased significantly during high workload and recovered completely after 15 min baseline perfusion in hearts with only 1% CK activity (8). Thus, this amount of CK is sufficient to synthesize and use PCr. It is possible that it takes a longer time to reach a new equilibrium for hearts with 2% CK activity. Based on our experience with high workload/recovery experiments, we have allowed sufficient time (30 min) for the PCr to be turned over 18 times before the ^{31}P NMR spectra were acquired after CK inhibition in the present study. Thus, our approach to calculate [MgADP] using CK reaction equilibrium should be valid under these experimental conditions.

Biochemical assays. Ventricular tissue (5–10 mg) was homogenized for 10 s at 4°C in potassium phosphate buffer containing 1 mM EDTA and 1 mM β-mercaptoethanol, pH 7.4 (final concentration of 5 mg tissue/ml). Aliquots were removed for assays of protein by the method of Lowry (13) using bovine serum albumin as the standard. Triton X-100 was then added to the homogenate at a final concentration of 0.1% for analysis of creatine kinase (CK) activity (14). The CK activity was measured in tissue homogenates at 30°C for each heart reported in this study. Treatment with 30, 90, and 120 μmol of IA inhibited the CK activity by 76, 95, and 98%, respectively, except as otherwise stated. The activities of the glycolytic enzymes, phosphofructokinase (PFK), glyceraldehyde 3-phosphate dehydrogenase (GAPdH) and lactate dehydrogenase (LDH), were analyzed as described in references 15–17. All enzyme activities are expressed as International Units (IU = μmol/min)/g of cardiac protein. All reagents used were at least analytical grade obtained from Sigma Chemical Co.

Measurement of $[Ca^{2+}]_c$. Indo-1 fluorescence was used to assess $[Ca^{2+}]_c$ on the surface of perfused hearts as described in references 18 and 19. This method is based on previous work that has identified and minimized potential sources of artifact in the indo-1 technique. Specifically, the effects of motion (18), autofluorescence (18, 19), unhydrolyzed indo-1 AM (20), tissue filter effect (18, 19), potential loading of indo-1 into endothelial cells (20), and noncytosolic compartmentation (21) have been accounted for and/or minimized. The ratio of indo-1 fluorescence at 385 and 456 nm during excitation at 350 nm was calibrated to calculate $[Ca^{2+}]_c$ as described in detail in references 18, 19, and 21. The rates of decline for the left ventricular pressure and $[Ca^{2+}]_c$ transient were assessed by the time constants of the monoexponential decline (τ_P and τ_{Ca}, respectively) as reported in reference 22. Indo-1 AM was obtained from Molecular Probes, Inc. (Eugene, OR).

Measurement of glycolytic rate. In a parallel experiment, hearts were first perfused and treated with either vehicle or 120 μmol of IA as described above. After the infusion of vehicle or IA was complete, hearts were switched to a recirculating buffer perfusion system at an identical perfusion pressure of 100 mmHg at 37°C. Glycolytic flux was determined by the addition of [5-^{3}H]glucose (DuPont-NEN Products, Boston, MA) to a total buffer volume of 100 ml (~ 14 μCi/mmol). Metabolism of [5-^{3}H]glucose through glycolysis leads to release of ^3H_2O at the enolase reaction, which is downstream of both the PFK and GAPdH catalyzed steps in glycolysis. The glycolytic rate was measured by quantifying the rate of ^3H_2O release to the perfusate as described by Saddick and Lopaschuk (23), and is expressed as μmol of glucose/min per g protein.

Statistical analysis. All results were expressed as mean±standard error of the mean. Comparisons among groups were made by ANOVA using the dose of IA as a grouping factor. When significant effects were observed, a two-tailed *t* test was used as a post hoc test. Measurements made before and after treatment with IA were compared by repeated measures ANOVA or paired *t* test. All statistical analyses were performed using Statview (Brainpower Inc., Calabasas, CA) and a value of $P < 0.05$ was considered significant. Lower probabilities were not reported.

Results

Fig. 1 shows representative ^{31}P NMR spectra and difference spectra for one heart before and after treatment with 120 μmol of IA and another heart before and after infusion with H_2O

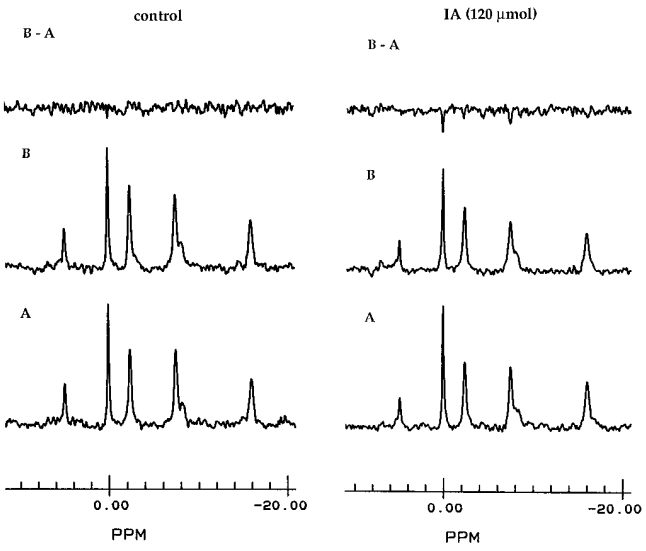

Figure 1. Representative ^{31}P NMR spectra for hearts before (*A*) and after (*B*) treatment with H_2O (*control*, *left*) or 120 μmol of IA (*right*). The difference spectra for each heart is shown (*B - A*).

Table I. ^{31}P NMR Measurements in Hearts Treated with Increasing Doses of IA

	Control	IA 30 μmol	IA 90 μmol	IA 120 μmol
PCr (mM)	16.3±0.9	14.3±0.9	12.0±0.7*	11.0±0.4*
ATP (mM)	11.1±0.9	10.7±0.4	10.4±0.3	9.9±0.5
ADP (μM)	58±7	73±8*	93±10*	107±9*
Pi (mM)	5.7±0.4	3.8±0.4	5.6±0.8	5.3±0.5
pH_i	7.11±0.01	7.09±0.01	7.10±0.01	7.08±0.01
n	7	6	6	6

Data are mean±SE; IA, iodoacetamide; PCr, phosphocreatine; pH_i, intracellular pH; *n*, number of hearts. *$P < 0.05$ versus control.

(control). Infusion of H_2O did not cause any changes in the spectra. With the highest dose of IA used in this study, there were no changes in the Pi or ATP peak areas, but there was a significant decrease in PCr peak area. The concentrations of high energy phosphates and pH_i for all hearts treated with increasing doses of IA are shown in Table I. There was an decrease in [PCr] in hearts treated with IA while [MgATP] and [Pi] were unchanged. There are two likely explanations for the absence of reciprocal change in [PCr] and [Pi] in hearts treated with IA. First, there may be some net efflux of intracellular Pi during the period of IA infusion (30 min) under the high flow condition, and second, part of the phosphate is trapped as the intermediate metabolites such as sugar phosphates. During high workload, a broad phosphomonoester peak appears in hearts treated with IA (not shown). The pH_i was not altered by IA in any groups.

Because [PCr] decreased in a dose-dependent fashion with increased [IA] and total [Cr] remained the same (8), a graded increase in free intracellular [MgADP] was found in hearts treated with increasing doses of IA ($P < 0.05$). Thus, inhibition of CK activity by IA induced an dose-dependent increase in free [MgADP] without any changes in [MgATP], [Pi], or pH_i.

Table II shows the left ventricular pressure and heart rate in these control and CK-inhibited hearts. The left ventricular systolic pressure (LVSP) and the spontaneous heart rate were similar in all groups. In contrast, LVEDP was increased in CK-inhibited hearts in a dose-dependent fashion ($P < 0.05$), suggesting a diastolic dysfunction in these hearts.

The relationship between intracellular free [MgADP] and the development of diastolic dysfunction is shown in two ways. First, a positive relationship between the increase in [MgADP] and the increase in LVEDP was found using data collected at the end of IA treatment (Fig. 2). Second, the temporal relationship between [MgADP] and LVEDP during the period of

Table II. Effect of IA on Left Ventricular Pressure

	Control	IA 30 μmol	IA 90 μmol	IA 120 μmol
LVSP (mmHg)	115±6	95±6	102±4	102±5
LVEDP (mmHg)	7±1	10±1*	13±2*	16±2*
Heart rate (min^{-1})	282±8	273±11	265±6	282±5
n	13	6	6	12

Data are mean±SE; IA, iodoacetamide; LVSP, left ventricular systolic pressure; LVEDP, left ventricular end diastolic pressure; *n*, number of hearts. *$P < 0.05$ versus control.

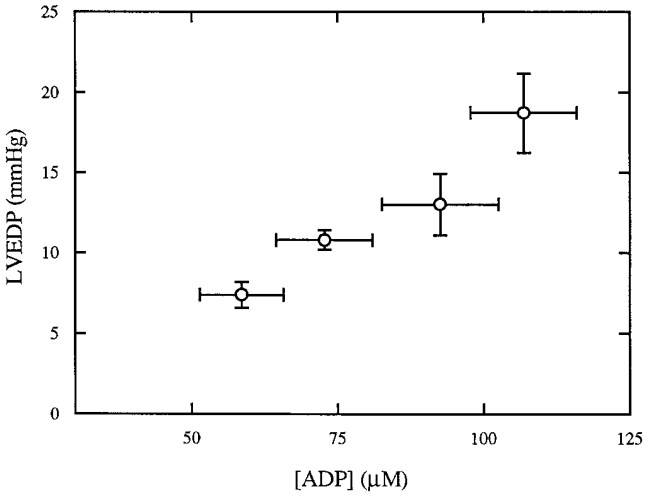

Figure 2. The relationship between the increase in [MgADP] and the increase in LVEDP using data collected from four groups of hearts treated with 0, 30, 90, or 120 μmol IA. Data are shown as mean±SEM.

IA infusion was recorded for another group of control and IA-treated hearts (Fig. 3). For control hearts, infusion of the vehicle did not cause significant changes either in [MgADP] or LVEDP during the 30-min period (Fig. 3, *left*). In contrast, for hearts treated with IA, there was a parallel increase in [MgADP] and LVEDP during the infusion period (Fig. 3, *right*). Thus, we found concomitant increases in [MgADP] and LVEDP in these hearts in the absence of any changes in [MgATP], [Pi], or pH_i.

To compare the impairment of diastolic function and the changes in $[Ca^{2+}]_c$, LVEDP, and τ_P (Fig. 4 *A*) are presented together with diastolic $[Ca^{2+}]_c$ and τ_{Ca} (Fig. 4 *B*). These data were obtained simultaneously in the same heart before and after application of a vehicle or IA. In control hearts, infusion of H_2O as a vehicle did not cause any significant change in LVEDP or $[Ca^{2+}]_c$. In hearts treated with IA, a threefold increase in LVEDP was accompanied by a 38% increase in τ_P, demonstrating significant diastolic dysfunction. However, diastolic $[Ca^{2+}]_c$ was increased by only 16% in these hearts ($P < 0.05$), and τ_{Ca} was not significantly changed by IA.

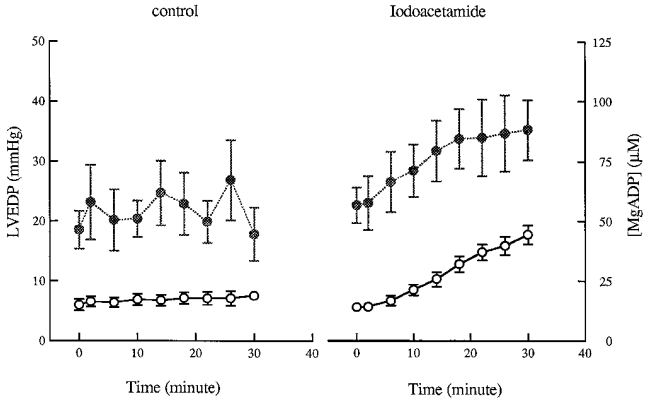

Figure 3. The temporal relationship of [MgADP] (*filled symbols*) and LVEDP (*open symbols*) during 30 min infusion of H_2O as a vehicle (*control*, $n = 5$; *left*) or iodoacetamide ($n = 7$; *right*). The CK activity was inhibited by 92% at the end of 30 min IA infusion. Data are shown as mean±SEM.

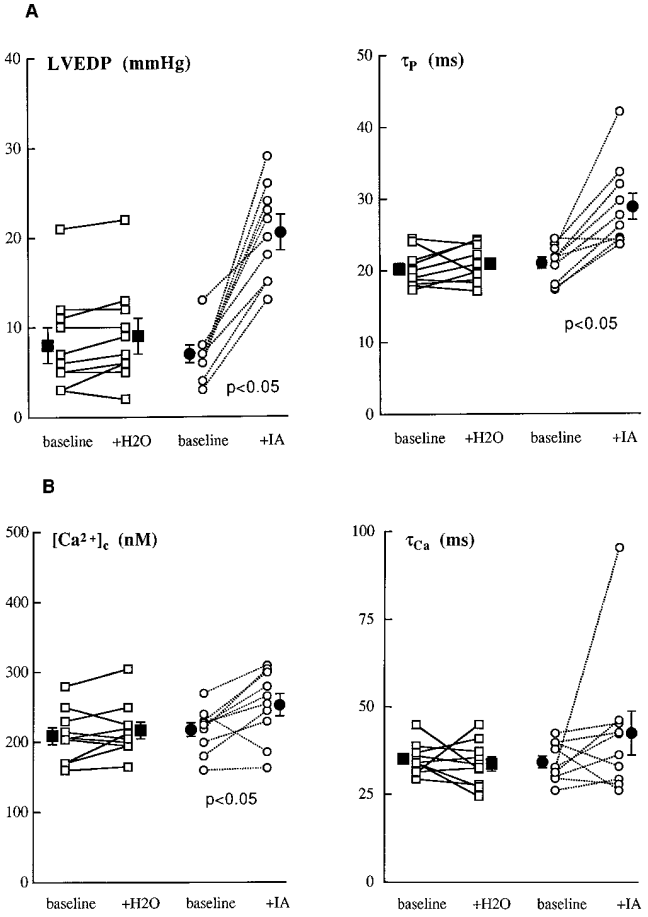

Figure 4. LVEDP and τ_P (Fig. 3 *A*) are compared to the diastolic cytosolic calcium concentration ($[Ca^{2+}]_c$) and τ_{Ca} (Fig. 3 *B*) simultaneously obtained from each individual heart before (baseline) and after treatment with either vehicle ($+H_2O$) or iodoacetamide (+IA). Each open square represents data obtained from a heart in the control group, and each open circle represents data obtained from a heart in IA group. The filled symbols are mean±SE for each measurement.

The effect of IA on the activities of glycolytic enzymes is shown in Table III. The activities of PFK and lactate dehydrogenase (LDH) were unchanged while there was a dose-dependent inhibition of GAPdH activity (~ 80%, $P < 0.05$) in hearts treated with IA. The glycolytic rate, measured in hearts treated with the highest dose of IA (120 μmol), was not different from that of the controls (Table III). In fact, the remaining 20% GAPdH activity (152 μmol/min per g protein) still exceeds the measured glycolytic rate in the control hearts (2.61 μmol/min per g protein) by more than 50 times. Taken together, these results indicate that glycolysis is unrestricted even in hearts with partially inhibited GAPdH activity.

Discussion

Sarcomere relaxation depends on dissociation of actin and myosin. The interaction of actin and myosin is regulated in a complex fashion by a number of factors, including intracellular

Table III. Effect of IA on Glycolytic Enzyme Activities and Glycolytic Rate

	Control	IA 30 μmol	IA 90 μmol	IA 120 μmol
PFK (IU/g protein)	167±6	151±16	148±27	161±11
GAPdH (IU/g protein)	715±61	592±51	291±29*	152±22*
LDH (IU/g protein)	3034±181	3121±247	3105±283	2881±134
Glycolytic rate (μmol/min per g protein)	2.42±0.18			2.62±0.30
n	13	6	6	12

Data are mean±SE; IA, iodoacetamide; PFK, phosphofructokinase; GAPdH, glyceraldehyde 3-phosphate dehydrogenase; LDH, lactate dehydrogenase; *n*, number of hearts. $*P < 0.05$ vs. control.

[MgATP] as well as MgATP hydrolysis products [MgADP] and [Pi], pH_i, and $[Ca^{2+}]_c$. Studies on diastolic dysfunction in ischemia reperfusion injury also suggest that MgATP derived from glycolysis might play a role in sustaining diastolic relaxation (9, 24). The novel finding in this study is that an impairment of myocardial relaxation in an intact heart could be induced by increasing cytosolic [MgADP] with minimal or no detectable changes in other candidate contributors for diastolic dysfunction under well-oxygenated conditions. This observation indicates that failure to maintain a low [MgADP], even at a normal level of [MgATP] and [Pi], may lead to slowed cross-bridge cycling and, consequently, to impaired myocardial relaxation.

The experimental strategy. The contribution of an altered intracellular high energy phosphate content to diastolic dysfunction has been well documented in myocardial injury by ischemia or hypoxia (25). However, it has been difficult technically to distinguish the contributions from MgATP depletion, MgADP or Pi accumulation, or changes in pH_i to diastolic dysfunction under these conditions. To define the unique contribution of free [MgADP], we used a low dose of IA to selectively inhibit CK activity in the heart. Since the CK reaction ($PCr + MgADP + H^+ <==> MgATP + Cr$) is one of the major mechanisms for maintaining a low free [MgADP], inhibition of this reaction leads to increased free [MgADP] in the cell. The specificity of low dose IA for inhibition of creatine kinase has been tested in our previous studies (7, 8). No functional changes in the primary MgATP synthesis (indices of mitochondrial respiration and the adenylate kinase reaction) and utilization (myofibrillar ATPase) pathways have been found in hearts treated with up to 140 μmol of IA (7). Furthermore, myocardial oxygen consumption and baseline contractile function were unaltered in isolated perfused hearts treated with IA (7, 8). Measurements by ^{31}P NMR spectroscopy have confirmed that applying a small amount of IA to inhibit the CK activity does not alter [MgATP] at baseline workload in our previous and current studies (7, 8). Taken together, using a low dose of IA, we were able to achieve an inhibition of the CK reaction without affecting oxidative phosphorylation or glycolysis which allowed a dose-dependent increase in intracellular free [MgADP] without altering [MgATP], [Pi], or pH_i in an isolated perfused rat heart. Thus, this strategy provides an opportunity to test the hypothesis that an acute increase in intracellular free [MgADP] alone is sufficient to induce diastolic dysfunction in an intact heart.

[MgADP] and diastolic dysfunction. In hearts treated with increasing doses of IA, we observed up to a threefold increase in LVEDP. A 38% increase in τ_P was also found in IA-treated hearts. Both findings showed the development of diastolic dysfunction in these hearts. It needs to be emphasized that these changes occurred in the absence of alterations in intracellular [MgATP], [Pi], and pH_i. Instead, we found a positive relationship between the increase in [MgADP] and the increase in LVEDP. Furthermore, the increases in [MgADP] and LVEDP were parallel during the period of IA infusion. These results imply that increased free [MgADP] plays an important role in the development of diastolic dysfunction in the intact heart.

The mechanism by which MgADP impairs myocardial relaxation has been suggested but has not been completely understood. Previous studies using isolated contractile proteins or skinned fibers have suggested that the detachment of MgADP from the actomyosin complex may be the rate limiting step in cross-bridge cycling, and increase in free [MgADP] may significantly slow down the rate of MgATP binding to actomyosin complex, resulting in impaired relaxation (6, 26–28). However, in most of these experiments, [MgADP] was in mM range which is much higher than typical physiological levels in the heart. A study using [MgADP] over the physiological range did not find any alterations in force development of skinned skeletal muscle fibers, but the effect of [MgADP] on fiber relaxation was not investigated (29). It is, therefore, difficult to extrapolate results from that study to the relationship of [MgADP] and LVEDP observed in this study. It is also possible that different types of striated muscle may yield different results. In support of this, a study showed that cardiac contractile proteins are more sensitive to [MgADP] than skeletal muscle proteins (29). Consistent with the in vivo results presented here, this same study showed that the sliding velocity of actin filaments on cardiac myosin was significantly reduced by adding MgADP at a concentration as low as 25 μM in the presence of 2 mM of MgATP (30).

More recent studies using smooth muscle actomyosin complex showed that a structural change in myosin occurred during transition from MgADP-bound state to rigor state (31). It is likely that diastolic dysfunction caused by increased [MgADP] observed in the present study of intact heart is distinct from the myocardial contracture observed in the rigor state, and independent mechanisms may be responsible for impairment of relaxation when [MgATP] decreases. This supports our previous and current observations that impairment of relaxation can be induced by an increase in [MgADP] even though MgATP is not depleted (25, 32). The increase in free [MgADP] was accompanied by a decrease in [PCr], raising the possibility that PCr directly affects relaxation. However, PCr is not a substrate of any reaction except the CK reaction. Therefore, it is very likely the only way that changes in the CK activ-

ity and [PCr] alter contraction or relaxation is via the changes in [MgADP] and [MgATP].

$[Ca^{2+}]_c$ and diastolic dysfunction. It is widely recognized that cytosolic calcium overload can lead to diastolic dysfunction, especially in ischemic and hypertrophied myocardium. In the rat, the rate of cytosolic calcium decline during diastole is primarily determined by calcium reuptake by the sarcoplasmic reticulum (SR) Ca^{2+}-ATPase (33). Our previous study has shown a decrease in the driving force for ATPase reactions (free energy release from MgATP hydrolysis) in hearts treated with IA (8). Since the calcium reuptake by SR Ca^{2+}-ATPase requires the highest driving force among all the other ATPase reactions (34), it is essential to define the contribution of $[Ca^{2+}]_c$ to the diastolic dysfunction observed in this study.

Our data suggest that changes of calcium handling at baseline contractile function cannot explain the diastolic dysfunction observed after IA treatment. Specifically, τ_{Ca} was not changed by IA treatment, even though there was a 38% increase in τ_P. Although there was a 16% increase in diastolic $[Ca^{2+}]_c$, this increase was modest relative to the three-fold increase in LVEDP. We thus conclude that changes of calcium handling at baseline did not play a major role in the development of diastolic dysfunction in this model.

Glycolysis and diastolic dysfunction. It has been suggested that MgATP derived from glycolysis in the cytosol is particularly useful for preventing the development of diastolic dysfunction in postischemic myocardium (35). This finding implies that glycolysis may be a protective mechanism for maintaining normal diastolic function. We found that the in vitro maximal activity of GAPdH, one of the enzymes in glycolytic pathway, was inhibited by up to 80% in hearts treated with IA. Therefore, the glycolytic rate was directly measured in hearts which received the highest dose of IA (120 μmol). Compared to the controls, the rate of glycolysis was not different in IA-treated hearts, indicating that the diastolic dysfunction in these hearts was not due to reduced MgATP synthesis from glycolysis. These results also suggest that even large reductions in GAPdH activity in a well-oxygenated heart does not limit glycolytic flux through this portion of the pathway.

Limitations of the study. The intracellular free [MgADP] calculated in this study gives an average cytosolic [MgADP] and may not be equal to the free [MgADP] at the actomyosin cross-bridge. Nevertheless, it is likely that changes in free [MgADP] at the myofibril are determined in large part by the changes in overall intracellular free [MgADP]. It is difficult to compare our results in the intact heart with results obtained from skinned muscle fibers since the disruption of the intracellular environment may have quantitatively changed the actomyosin mechanics in skinned muscle. Moreover, due to the presence of CK and adenylate kinase associated with myofibrils, which function to convert MgADP to MgATP and/or AMP, the free [MgADP] for skinned muscle fibers cannot be quantitatively determined by the amount of MgADP applied to the fibers. Thus, we can compare the relationship between [MgADP] and the development of diastolic dysfunction found in this experiment to previously reported results in skinned muscle studies only qualitatively.

The indo-1 fluorescence study in perfused heart may be limited by the method that collects the signals only from several outer layers of the epicardium. There is a difference between epicardial and endocardial $[Ca^{2+}]_c$. It is possible that inhomogeneity in $[Ca^{2+}]_c$ occurred in IA treated hearts and was not detected by our measurement. However, it has been shown that the difference between epicardial and endocardial $[Ca^{2+}]_c$ is constant in well-perfused hearts and is not altered by reducing coronary flow up to 50% (19). Thus, it is likely that changes in epicardial $[Ca^{2+}]_c$ accurately represent directional changes in $[Ca^{2+}]_c$ of the whole heart to large extent in our conditions.

In summary, using a low dose of IA to selectively inhibit CK activity in isolated perfused rat hearts, we observed a significant impairment of diastolic function. The rate of glycolysis was not changed by treatment with IA in spite of $\sim$ 80% inhibition of GAPdH activity. The modest increase in $[Ca^{2+}]_c$ found in these hearts cannot explain the severity of diastolic dysfunction. IA caused a dose-dependent increase in intracellular free [MgADP] without changing [MgATP], [Pi], or pH_i. Furthermore, a close relationship between the development of diastolic dysfunction and the increase in free [MgADP] was observed in these hearts, indicating that increased free [MgADP] is a significant contributor to diastolic dysfunction independent of any changes in other regulators of cross-bridge cycling.

Acknowledgments

We would like to thank Dr. Liang Zhao for his thoughtful input and stimulating discussion of this study. The skillful technical assistance of Ilana Reis is greatly appreciated.

This study was supported by grants from the National Institutes of Health (HL 52350 to J.S. Ingwall, Individual National Research Service Award HL08973 to J.M. Halow), American Heart Association (Grant-in-Aid 95-220 to S.A. Camacho), and American Heart Association California Affiliate (Grant-in-Aid 94-6930 to S.A. Camacho). M.E. Christe is a Howard Hughes research fellow; M. Spindler is a research fellow of the Deutsche Forschungsgemeinschaft.

References

1. Taylor, E.W. 1979. Mechanism of actomyosin ATPase and the problem of muscle contraction. *CRC Crit. Rev. Biochem.* 7:103–164.

2. Eisenberg, E., and L.E. Greene. 1980. The relation between muscle physiology and muscle biochemistry. *Annu. Rev. Physiol.* 42:293–309.

3. Rayment, I., H.M. Holden, M. Whittaker, C.B. Yohn, M. Lorenz, K.C. Holmes, and R.A. Milligan. 1993. Structure of the actin-myosin complex and its implications for muscle contraction. *Science (Wash. D.C.).* 261:58–65.

4. Hibberd, M.G., and D.R. Trentham. 1986. Relationship between chemical and mechanical events during muscular contraction. *Annu. Rev. Biophys. Chem.* 15:119–161.

5. Dantzig, J.A., Y.E. Goldman, N.C. Millar, J. Laktis, and E. Homsher. 1992. Reversal of the cross-bridge force-generating transition by photogeneration of phosphate in rabbit psoas muscle fibers. *J. Physiol (Lond.).* 451:247–278.

6. Cooke, R., and E. Pate. 1985. The effects of ADP and phosphate on the contraction of muscle fibers. *Biophys. J.* 48:789–798.

7. Hamman, B.L., J.A. Bittl, W.E. Jacobus, P.D. Allen, R.S. Spencer, R. Tian, and J.S. Ingwall. 1995. Inhibition of creatine kinase reaction decreases the contractile reserve of isolated rat hearts. *Am. J. Physiol.* 269:H1030–H1036.

8. Tian, R., and J.S. Ingwall. 1996. Energetic basis for reduced contractile reserve in isolated rat hearts. *Am. J. Physiol.* 270:H1207–H1216.

9. Kagaya Y., E.O. Weinberg, N. Ito, T. Mochizuki, W.H. Barry, and B.H. Lorell. 1995. Glycolytic inhibition: effects on diastolic relaxation and intracellular calcium handling in hypertrophied rat ventricular myocytes. *J. Clin. Invest.* 95:2766–2776.

10. Polimeni, P.I., and S.I. Buraczewski. 1988. Expansion of extracellular tracer spaces in the isolated heart perfused with crystalloid solution: expansion of extracellular space, transsarcolemmal leakage, or both? *J. Mol. Cell. Cardiol.* 20:15–22.

11. Lawson, J.W.R., and R.L. Veech. 1979. Effect of pH and free Mg^{2+} on the K_{eq} of the creatine kinase reaction and other phosphate hydrolyses and phosphate transfer reactions. *J. Biol. Chem.* 254(14):6528–6537.

12. Tian, R., P. Gaudron, S. Neubauer, K. Hu, and G. Ertl. 1995. Alterations of performance and oxygen utilization in chronically infarcted rat hearts. *J. Mol. Cell. Cardiol.* 28:321–330.

13. Lowry, O.H., M.J. Rosebrough, A.L. Farr, and R.J. Randall. 1951. Protein measurement with the Phenol Folin reagent. *J. Biol. Chem.* 193:265–275.

14. Rosalki, S.P. 1967. An improved procedure for serum creatine phosphokinase determination. *J. Lab. Clin. Med.* 69:696–705.

15. Oblinger, M.M., L.G. Foe, D. Kwiatkoska, and R.G. Kemp. 1988. Phosphofructokinase in the rat nervous system: regional differences in activity and characteristics of axonal transport. *J. Neurosci. Res.* 21:25–34.

16. Bergmeyer, H.U. 1974. *In* Methods of Enzymatic Analysis. H.U. Bergmeyer and K. Gawehn, editors. Academic Press Inc., Orlando, FL. vol. 1:466–467.

17. Bernstein L.H., and J. Everse. 1975. Determination of the isoenzyme levels of lactate dehydrogenase. *Methods Enzymol.* 41:47–49.

18. Brandes, R., V.M. Figueredo, S.A. Camacho, A.J. Baker, and M.W. Weiner. 1993*a.* Quantitation of cytosolic [Ca^{2+}] in whole perfused rat hearts using indo-1 fluorometry. *Biophys. J.* 65:1973–1982.

19. Brandes, R., V.M. Figueredo, S.A. Camacho, A.J. Baker, and M.W. Weiner. 1993*b.* Investigation of factors affecting fluorometric quantitation of cytosolic [Ca^{2+}] in perfused hearts. *Biophys. J.* 65:1983–1993.

20. Figueredo, V.M., R. Brandes, M.W. Weiner, B.M. Massie, and S.A. Camacho. 1993. Endocardial versus epicardial differences of intracellular free calcium under normal and ischemic conditions in perfused rat hearts. *Circ. Res.* 72: 1082–1090.

21. Schreur, J.H.M., V.M. Figueredo, M. Miyamae, D.M. Shames, A.J. Baker, and S.A. Camacho. 1996. Cytosolic and mitochondrial [Ca^{2+}] in whole heart using indo-1 AM: effects of high extracellular Ca^{2+}. *Biophys. J.* 70:2571–2580.

22. Chang, C.K., J.H.M. Schreur, M.W. Weiner, and S.A. Camacho. 1996. Slowing of Ca^{2+} transient decline is an early manifestation of pressure overload hypertrophy in rat hearts. *Am. J. Physiol.* 271:H228–234.

23. Saddik, M., and G.D. Lopaschuk. 1991. Myocardial triglyceride turnover and contribution to energy substrate utilization in isolated working rat hearts. *J. Biol. Chem.* 266:8162–8170.

24. Cunningham, M.J., C.S. Apstein, E.O. Weinberg, W.M. Vogel, and B.H. Lorell. 1990. Influence of glucose and insulin on the exaggerated diastolic and systolic dysfunction of hypertrophied rat hearts during hypoxia. *Circ. Res.* 66: 406–415.

25. Bernard, M., S. Neubauer, and J.S. Ingwall. 1994. On the relations among ATP hydrolysis, cation accumulation and diastolic dysfunction. *In* Diastolic Relaxation of the Heart. B.H. Lorell, and W. Grossman, editors. Kluwer Academic Publishers, Norwell, MA. 73–78.

26. Siemankowski, R.F., M.O. Wiseman, and H.D. White. 1985. ADP dissociation from actomyosin subfragment 1 is sufficiently slow to limit the unloaded shortening velocity in vertebrate skeletal muscle. *Proc. Natl. Acad. Sci. USA.* 82:658–662.

27. Danztig, J.A., M.G. Hibberd, D.R. Trentham, and Y.E. Goldman. 1991. Cross-bridge kinetics in the presence of MgADP investigated by photolysis of caged ATP in rabbit psoas muscle fibers. *J. Physiol.* 432:639–680.

28. Ma, Y.Z., and E.W. Taylor. 1994. Kinetics mechanism of myofibril ATPase. *Biophys. J.* 66:1542–2553.

29. Chase P.B, and M.J. Kushmerick. 1995. Effect of physiological ADP concentration on contraction of single skinned fibers from rabbit fast and slow muscles. *Am. J. Physiol.* 268:C480–C489.

30. Yamashita H., M. Sata, S. Sugiura, S. Momomura, T. Serizawa, and M. Iizuka. 1994. ADP inhibits the sliding velocity of fluorescent actin filaments on cardiac and skeletal myosins. *Circ. Res.* 74:1027–1033.

31. Whittaker M., E.M. Wilson-Kubalek, J.E. Smith, L. Faust, R.A. Milligan, and H.L. Sweeney. 1995. A 35-A movement of smooth muscle myosin on ADP release. *Nature (Lond.).* 378:748–751.

32. Tian, R., L. Nascimben, E.O. Weinberg, J.S. Ingwall, and B.H. Lorell. 1995. Altered regulation of [ADP] in cardiac hypertrophy severely impairs diastolic function. *Circulation.* 92(suppl. 1):I–658 (Abstr.)

33. Bers, D.M. 1991. Sarcoplasmic reticulum Ca^{2+} uptake, content and release. *In* Excitation-Contraction Coupling and Cardiac Contractile Force. Kluwer Academic Publishers Group, Dordrecht, The Netherlands. 93–118.

34. Kammermeier, H. 1987. High energy phosphate of the myocardium: concentration versus free energy change. *Basic Res. Cardiol.* 82:31–36.

35. Owen, P., S. Dennis, and L.H. Opie. 1990. Glucose flux rate regulates onset of ischemic contracture in globally underperfused rat hearts. *Circ. Res.* 66: 344–354.