Role of Phospholamban in Cyclic GMP Mediated Signaling in Cardiac Myocytes

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Key Words

Cardiac myocyte function • Natriuretic peptides • Nitric oxide • Cyclic GMP • Phospholamban

Abstract

We tested the hypothesis that the negative functional effects of cyclic GMP on cardiac myocytes were mediated through phospholamban (PLB) and activation of sarcoplasmic reticulum Ca^{2+} -ATPase. Using ventricular myocytes from wild type (WT, $n=10$) and PLB knockout (PLB-KO, $n=10$) mouse hearts, functional changes were measured using a video edge detector at baseline and after 10^{-6} , 10^{-5} M 8-bromo-cyclic GMP (cGMP), 10^{-8} , 10^{-7} M C-type natriuretic peptide (CNP), or 10^{-6} , 10^{-5} M S-nitroso-N-acetylpenicillamine (SNAP, nitric oxide donor). Changes in cytosolic Ca^{2+} concentration were assessed in fura 2-loaded WT and PLB-KO myocytes. Cyclic GMP dependent phosphorylation analysis was also performed in WT and PLB-KO myocytes. 8-bromo-cGMP 10^{-5} M caused a significant decrease in %shortening ($3.6\pm 0.2\%$ to $2.3\pm 0.1\%$) in WT, but little change in PLB-KO myocytes ($3.4\pm 0.1\%$ to $3.2\pm 0.2\%$). Similarly, CNP and SNAP reduced %shortening of WT, but not PLB-KO myocyte. Changes in other contractile

parameters such as maximum rate of shortening and relaxation were consistent with the changes in % shortening. Intracellular Ca^{2+} transients changed similarly to cell contractility in WT and PLB-KO myocytes treated with cGMP and CNP; i.e. Ca^{2+} transients decreased with cGMP or CNP in WT myocytes, but were unchanged in PLB-KO myocytes. cGMP dependent phosphorylation analysis showed that some proteins were phosphorylated by cGMP to a lesser extent in PLB-KO compared with WT myocytes, suggesting impaired cGMP-kinase function in PLB-KO cardiac myocytes. These results indicated that cGMP-induced reductions in cardiac myocyte function were at least partially mediated through the action of phospholamban.

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Introduction

Cyclic GMP, produced in response to nitric oxide (soluble guanylyl cyclase activator) and natriuretic peptides (particulate guanylyl cyclase activator), is an important regulator of myocardial function. It has been shown that cyclic GMP reduces myocardial metabolism,

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inotropy and function [1]. Elevation in the level of cyclic GMP decreases myocyte oxygen consumption and myocyte contraction in rabbit, mouse and canine hearts and cardiac ventricular myocytes [2, 3]. The negative effects of cyclic GMP are mainly mediated through the cyclic GMP-dependent protein kinase and this could reduce intracellular Ca^{2+} by activation of the sarcoplasmic reticulum Ca^{2+} -ATPase (SERCA) [4, 5]. Cyclic GMP mediated signaling may also involve phosphorylation of ryanodine receptors (RyRs) by cyclic GMP-dependent protein kinase and/or indirectly regulate RyRs by changing the cytosolic levels of RyRs through cADP-ribose [6]. The cyclic GMP-signaling pathway may also be mediated by protein kinase-independent interaction with other molecules in the cell, such as cyclic GMP-gated cation channels and certain phosphodiesterases [7-9].

The regulation of intracellular calcium is one of the major determinants of cardiac function. Muscle contraction is initiated by calcium released from sarcoplasmic reticulum and relaxation is initiated by calcium being pumped back into sarcoplasmic reticulum by SERCA [10]. In heart, the activity of SERCA is regulated by phospholamban (PLB), an integral sarcoplasmic reticulum phosphoprotein, or by phosphorylation through Ca^{2+} /calmodulin dependent protein kinase II [11, 12]. Unphosphorylated PLB inhibits SERCA activity by decreasing the affinity of the ATPase for Ca^{2+} . Phosphorylation of PLB relieves SERCA inhibition and increases SERCA activity [13]. It had been shown that cyclic GMP-induced reductions in myocyte function were partially mediated through the action of SERCA [5]. The interaction between SERCA2a and phospholamban can be disrupted by Ca^{2+} binding to SERCA2a [14].

Previous studies in our laboratory suggested that cyclic GMP signaling affects myocyte function, in part, through phosphorylation of phospholamban, which regulates SERCA activity [2, 5]. In the current study, we used a genetic approach using phospholamban knockout (PLB-KO) mouse myocytes to test the hypothesis that the negative functional effects of cyclic GMP on cardiac myocytes were mediated through cyclic GMP-dependent regulation of phospholamban (PLB) and subsequent changes in intracellular calcium transients. Using activators of the particulate and soluble forms of guanylyl cyclase, we observed functional changes and calcium transients of control wild-type (WT) and PLB-KO myocytes in response to these reagents. The cyclic GMP dependent phosphorylation pattern was also investigated in WT and PLB-KO myocytes.

Material and Methods

Cell Dissociation

This investigation was conducted in accordance with the Guide for the Care of Laboratory Animals (DHHS Publication No. 85-23, Revised 1996) and was approved by the Institutional Animal Care and Use Committee. PLB homozygous knockout mice (PLB-KO, strain 0027) were purchased from Harlan Sprague Dawley (Indianapolis, Indiana). Wild type mice were from Jackson Laboratory (Bar Harbor, ME, 8-10 weeks old, either sex).

Ventricular myocytes were isolated from mouse hearts ($n=10$ each group) as previously described [15]. Briefly, the mice were anesthetized with an intraperitoneal injection of pentobarbital (60 mg/kg) and the heart was removed. The aorta was quickly cannulated, and the heart was subsequently mounted on a Langendorff perfusion apparatus. Retrograde perfusion was performed for 5min at 40 mm Hg constant pressure with modified calcium free minimal essential medium (MEM) supplemented with 10 mM taurine, 2 mM L-glutamic acid, and 20 mM HEPES, pH 7.35. Then the heart was perfused with MEM containing 0.1% type II collagenase (Worthington, Freehold, NJ) for 10min. The perfusion media was maintained at 37°C and equilibrated with a water-saturated gas mixture (95% O_2 -5% CO_2).

After collagenase digestion, the heart was removed from the apparatus, and the ventricles were lacerated into small pieces. The tissue was subsequently pipetted in the 0.1% collagenase solution until all the large pieces were dispersed in cell suspension. The supernatant containing isolated myocytes was then transferred to a 15-ml centrifuge tube. After being washed in MEM supplemented with 0.5% BSA, cells were collected. Myocyte viability was assessed by maintenance of a rod-shaped morphology and was between 50-80%. The cells prepared for functional studies and calcium measurements were subjected to calcium reintroduction. Finally, the myocytes were suspended in the media supplemented with 0.5% BSA and 1 mM CaCl_2 at room temperature.

Myocyte functional measurements

Functional measurements were obtained on individual ventricular myocytes. Cells were suspended in 2 ml of 2 mM Ca^{2+} MEM solution maintained at 37°C in a chamber that was fitted onto the stage of an inverted light microscope (Zeiss Axiovert 125, Carl Zeiss). Two platinum wires were inserted into two parallel sides of the chamber and were used to pace the myocytes by electric field stimulation (1 Hz, 5 msec duration, voltage 10% above threshold, and polarity altered with each pulse). Unloaded shortening of random selected cardiac myocytes was measured on-line using a video edge-detector system (Crystal Biotech, model VED-114, Data Sciences International) and a camera, which detected the change of the position of both edges of the cell. Data were collected continuously. The output of the video edge detector was fed into a television monitor and a desktop computer, which then analyzed the data. These data were used to calculate the reported functional parameters. Cells used to determine the

functional parameters were healthy and could react to different reagents throughout the course of the experiment. Untreated cells continued to contract at a constant level over the time course of the experiment.

Experimental Protocol

WT and PLB-KO mice myocytes were used for functional measurements. Three groups of myocytes from each strain were used in the following protocols. In all groups, myocytes in appropriate concentration were suspended in a chamber with 2 ml of MEM containing 2 mM Ca^{2+} and 0.5% BSA. After a 3-min stabilization period paced with electrical field stimulation, baseline contraction data for an individual myocyte were recorded. At 3-min intervals, reagents were added to the medium and allowed to diffuse to the cell during which cell contractility was measured. In the first group, 8-Br-cGMP (8-Bromoguanosine 3':5' cyclic monophosphate) was added at concentrations of 10^{-6} , 10^{-5} M. In the second group, C-type natriuretic peptide (CNP, Sigma) was added at concentrations of 10^{-8} , 10^{-7} M. In the third group, SNAP (S-nitroso-N-acetylpenicillamine (SNAP) 10^{-6} , 10^{-5} M was added to myocytes.

A minimum of 10 consecutive contractions were used for each data point. For each protocol at least 3 cells in each animal were repeatedly measured. Measurements obtained include resting cell length, absolute cell shortening, maximal rate of shortening, rate of cell relaxation, and calculated percentage of cell shortening.

Intracellular $[\text{Ca}^{2+}]$ Measurements

For the measurement of intracellular Ca^{2+} concentration, the fluorescent Ca^{2+} indicator, fura-2, was used. Ventricular myocytes were incubated with 2 μM fura-2 acetoxymethyl ester (fura-2 AM, Molecular Probes, Eugene, OR) in MEM solution containing 0.5% BSA and 1 mM CaCl_2 at room temperature for 1 hour. Unincorporated fura-2 AM was removed by washing the myocytes twice in fura-2 AM-free MEM solution.

The fura-loaded cells were suspended in 2 ml of 2 mM Ca^{2+} MEM solution containing 0.5% BSA maintained at 37°C in a chamber that was fitted onto the stage of a Nikon inverted microscope (TS100). Ventricular myocytes were paced by electric field stimulation at 1 Hz, 5 ms duration. A dual excitation spectrofluorometer was used to record fluorescence emissions (480–520 nm) elicited from exciting wavelengths at 360 and 380 nm and measurements were recorded using the Ionoptix Soft Edge and Fluorescence System (Ionoptix, Milton, MA). Qualitative changes in $[\text{Ca}^{2+}]$ were determined from the ratio of the fluorescence intensity at 360/380 nm wavelengths. Each experimental protocol was performed in at least three myocytes from each animal and 3 animals from WT and PLB-KO mice were used.

Myocyte Extract Preparation

The myocyte suspension was centrifuged. The pellet was collected and frozen immediately in liquid nitrogen. The frozen cells were stored in an -80°C freezer until needed. Myocytes were homogenized for 15 s at 20,000 rpm (Polytron Homogenizer) in homogenizing buffer (0.25 M sucrose, 5 mM Tris pH 7.4, 1 mM MgCl_2) and centrifuged at 30,000 g at 4°C for 20 min. The

supernatant was collected and used as myocyte extract for phosphorylation reactions. Protein concentrations of each sample were determined using a spectrometer at 595 nm. BSA was used as a standard. Cell extract aliquots were stored at -80°C .

Cyclic GMP-dependent Protein Phosphorylation and Electrophoresis

All reactions were carried out in microfuge tubes at room temperature. Protein concentration of the myocyte extracts were equalized to 1 mg/ml. The reaction mixture includes 10 μl of protein extract, 4 mM 8-bromo-cGMP, KT5823 (a cyclic GMP protein kinase inhibitor) and 0.5 μl $\gamma\text{-}^{32}\text{P}$ -ATP (10 $\mu\text{Ci}/\mu\text{l}$, Amersham) with a total volume of 16 μl . The reaction was allowed to occur for fifteen min and was terminated by adding 16 μl of BioRad reducing sample buffer. The phosphoprotein samples were heated at 95°C for 5 min. Proteins were electrophoresed and separated by 12% SDS-PAGE using the BioRad mini-gel. The gel containing phosphoproteins was dried on chromatography paper and autoradiography was performed.

Statistics

Results are expressed as means $\pm$ SEM. A repeated measure analysis of variance (ANOVA) was used to compare variables measured in the experimental and control conditions. Duncan's test was used to compare difference between baseline and various treatments. In all cases, a value of $p < 0.05$ was accepted as significant.

Results

PLB-KO mice were similar to wild type mice. The base level of myocyte percent shortening and other functional timing parameters of WT and PLB-KO mice were not significantly different from each other.

Cyclic GMP, CNP, and SNAP significantly decreased cell percent shortening in wild type cardiac myocytes, but not in PLB-KO myocytes

The cyclic GMP analog 8-bromo-cGMP, CNP (a stimulator of particulate guanylyl cyclase) and SNAP (a donor of nitric oxide which activates soluble guanylyl cyclase) were used to examine changes in the effects of the cyclic GMP signaling system on myocyte function in WT and PLB-KO myocytes. The functional responses of myocytes from WT and PLB-KO were then measured and analyzed.

In WT myocytes, when 8-Bromo-cGMP (10^{-6} – 10^{-5} M) was added to cells in 3-min intervals, the percentage shortening was decreased in a concentration dependent manner (Figure 1). The percentage shortening was decreased significantly from $3.6 \pm 0.2\%$ at base level to $2.3 \pm 0.1\%$ when 10^{-5} M 8-bromo-cGMP was present.

Fig. 1. The effects of 8-Br-cGMP, CNP and SNAP on percentage shortening of ventricular myocytes from wild type and PLB-KO mice. In the first group, the myocytes from wild type and PLB-KO were treated with 8-Bromo-cGMP 10^{-6} , 10^{-5} M. The second group was treated with CNP 10^{-8} , 10^{-7} M. The third group was treated with SNAP 10^{-6} , 10^{-5} M. WT = wild type, cGMP = 8-Bromo-cGMP, CNP = C-type natriuretic peptide, cGMP = 10^{-5} M cGMP. * Significantly different from baseline. ** Significantly different from CNP 10^{-6} M. # Significantly different from PLB-KO.

Cyclic GMP decreased myocyte percentage shortening by 36%. Similarly, CNP reduced percent shortening from $3.7 \pm 0.1\%$ at base level to $2.5 \pm 0.1\%$ when 10^{-7} M CNP was added with a 32% decrease. SNAP also reduced percent shortening from $3.5 \pm 0.2\%$ at base level to $2.1 \pm 0.1\%$ when 10^{-5} M SNAP was added with a 39% decrease (Figure 1).

In contrast, PLB-KO myocyte percent shortening exhibited minimal change upon the addition of 8-bromo-cGMP ($3.4 \pm 0.1\%$ baseline to $3.2 \pm 0.2\%$ with 10^{-5} M 8-bromo-cGMP), CNP ($3.3 \pm 0.2\%$ baseline to $3.1 \pm 0.1\%$ with 10^{-7} M CNP) or SNAP ($3.6 \pm 0.2\%$ baseline to $3.2 \pm 0.2\%$ with 10^{-5} M SNAP) with no statistical differences (Figure 1).

Cyclic GMP, CNP, and SNAP significantly decreased maximum rate of shortening and maximum rate of relaxation in wild type cardiac myocyte but not in PLB-KO myocytes

Baseline maximum rate of shortening and relaxation were significantly higher in PLB-KO myocytes than that of wild type myocytes, Table 1. Treatment induced changes in myocyte maximum rate of shortening and relaxation were similar to myocyte percent shortening.

In WT myocytes, addition of 8-bromo-cGMP ($10^{-6, -5}$ M) decreased maximum rate of shortening and relaxation in a concentration dependent manner. The maximum rate of shortening was decreased significantly by 31% and maximum rate of relaxation was decreased 29% in the presence of 10^{-5} M 8-bromo-cGMP; CNP at 10^{-7} M decreased maximum rate of shortening by 23% and maximum rate of relaxation by 22%. SNAP 10^{-5} M reduced maximum rate of shortening by 39% and maximum rate of relaxation by 34% (Table 1). In contrast, PLB-KO myocytes exhibited only small non-significant changes in maximum rate of shortening and relaxation

	Maximum Rate of Shortening ($\mu\text{m}/\text{sec}$)		Maximum Rate of Relaxation ($\mu\text{m}/\text{sec}$)	
	WT	PLB-KO	WT	PLB-KO
Baseline	48.35 $\pm$ 5.85	78.74 $\pm$ 4.67	46.72 $\pm$ 5.09	78.98 $\pm$ 4.88
cGMP 10^{-6}M	42.12 $\pm$ 5.67	76.47 $\pm$ 5.55	42.83 $\pm$ 5.63	76.33 $\pm$ 5.29
cGMP 10^{-5}M	33.25 $\pm$ 2.39 * #	74.61 $\pm$ 5.29	33.36 $\pm$ 2.13 * #	74.11 $\pm$ 4.92
Baseline	46.72 $\pm$ 1.83 #	81.49 $\pm$ 4.03	46.38 $\pm$ 1.74 #	81.33 $\pm$ 3.72
CNP 10^{-8}M	39.73 $\pm$ 2.51 #	74.63 $\pm$ 3.96	40.84 $\pm$ 2.96 #	74.03 $\pm$ 3.88
CNP 10^{-7}M	35.81 $\pm$ 3.81 * #	76.45 $\pm$ 5.91	36.30 $\pm$ 3.48 * #	73.45 $\pm$ 5.90
Baseline	49.98 $\pm$ 4.13 #	87.26 $\pm$ 8.50	49.66 $\pm$ 4.15 #	89.07 $\pm$ 7.84
SNAP 10^{-6}M	40.35 $\pm$ 3.97 #	84.36 $\pm$ 7.30	41.80 $\pm$ 3.73 #	85.51 $\pm$ 6.48
SNAP 10^{-5}M	30.73 $\pm$ 2.71 * #	77.14 $\pm$ 8.30	32.97 $\pm$ 2.71 * #	76.26 $\pm$ 8.28

Table 1. Effects of cyclic GMP, CNP and SNAP on maximum rate of shortening and relaxation of cardiac myocytes from wild type and PLB-KO mouse hearts. WT: wild type; PLB-KO: phospholamban knock out; cGMP: 8-bromo-cGMP; CNP: C-type natriuretic peptide; SNAP: S-nitroso-N-acetyl-penicillamine. * Significantly different from baseline. # Significantly different from PLB-KO.

	TTP (0.1 x sec)		R50 (0.01 x sec)		R90 (0.1 x sec)	
	WT	PLB-KO	WT	PLB-KO	WT	PLB-KO
Baseline	4.1 $\pm$ 0.2	4.4 $\pm$ 0.1	9.3 $\pm$ 0.3	8.8 $\pm$ 0.7	3.0 $\pm$ 0.3	2.8 $\pm$ 0.2
cGMP 10^{-6}M	4.6 $\pm$ 0.3	4.4 $\pm$ 0.2	9.6 $\pm$ 0.5	8.3 $\pm$ 0.6	3.2 $\pm$ 0.3	3.0 $\pm$ 0.1
cGMP 10^{-5}M	4.6 $\pm$ 0.2	4.6 $\pm$ 0.2	8.9 $\pm$ 0.6	9.9 $\pm$ 0.8	3.4 $\pm$ 0.1	3.5 $\pm$ 0.3
Baseline	4.2 $\pm$ 0.3	4.5 $\pm$ 0.3	7.9 $\pm$ 0.4	7.8 $\pm$ 0.8	2.9 $\pm$ 0.1	2.8 $\pm$ 0.2
CNP 10^{-8}M	4.3 $\pm$ 0.2	4.5 $\pm$ 0.3	8.3 $\pm$ 0.4	8.5 $\pm$ 0.8	3.3 $\pm$ 0.3	3.0 $\pm$ 0.2
CNP 10^{-7}M	4.4 $\pm$ 0.2	4.6 $\pm$ 0.3	9.3 $\pm$ 0.6	9.2 $\pm$ 0.7	3.5 $\pm$ 0.2	3.0 $\pm$ 0.2
Baseline	4.1 $\pm$ 0.2	4.2 $\pm$ 0.2	8.2 $\pm$ 0.4	8.7 $\pm$ 0.2	3.2 $\pm$ 0.3	3.2 $\pm$ 0.1
SNAP 10^{-6}M	4.5 $\pm$ 0.2	4.3 $\pm$ 0.2	9.6 $\pm$ 0.6	8.4 $\pm$ 0.4	3.7 $\pm$ 0.3	3.8 $\pm$ 0.2
SNAP 10^{-5}M	4.6 $\pm$ 0.1	4.3 $\pm$ 0.2	10.4 $\pm$ 0.8	7.8 $\pm$ 0.8	4.0 $\pm$ 0.3	3.1 $\pm$ 0.2

Table 2. Effects of cyclic GMP, CNP and SNAP on TTP, R_{50} and R_{90} on cardiac myocytes from wild type and PLB-KO mouse hearts. WT: wild type; PLB-KO: phospholamban knock out; cGMP: 8-bromo-cGMP; CNP: C-type natriuretic peptide; SNAP: S-nitroso-N-acetyl-penicillamine. TTP: time to peak; R_{50} : time from peak contraction to 50% relaxation; R_{90} : time from peak contraction to 90% relaxation.

upon addition of 8-bromo-cGMP, CNP, or SNAP (Table 1).

Baseline values of other contractile parameters such as time to peak contraction (TTP), time from peak contraction to 50% relaxation (R_{50}) and to 90% relaxation (R_{90}) were similar between myocytes from WT and PLB-KO. These parameters did not change significantly in response to 8-bromo-cGMP, CNP or SNAP in myocytes from either group of mice (Table 2).

Intracellular Ca^{2+} transients changed similarly to myocyte contractility upon addition of 8-bromo-cGMP, CNP or SNAP

To test whether the effects of 8-bromo-cGMP, CNP or SNAP on myocyte contractility were correlated to Ca^{2+} transients, intracellular Ca^{2+} concentration was measured in myocytes from wild type and phospholamban knock-out mice in the absence or presence of 8-bromo-cGMP, CNP, or SNAP.

Fig. 2. The effects of 8-Br-cGMP, CNP and SNAP on intracellular Ca^{2+} transients in ventricular myocytes from wild type and PLB-KO mice. In the first group, the myocytes from wild type and PLB-KO were treated with 8-Bromo-cGMP, 10^{-5} M. The second group was treated with CNP, 10^{-7} M. The third group was treated with SNAP, 10^{-5} M. WT = wild type, cGMP = 8-Bromo-cGMP, CNP = C-type natriuretic peptide, cGMP = 10^{-5} M cGMP. * Significantly different from baseline.

Baseline myocyte intracellular Ca^{2+} levels in WT and PLB-KO appeared to be similar (93.3 ± 9.6 nM (63 WT cells from 3 rabbits) vs. 93.0 ± 8.3 nM PLB-KO (64 cells from 3 rabbits)). As shown in Figure 2, 8-bromo-cGMP 10^{-5} M significantly decreased the amplitude of the Ca^{2+} transients in WT myocytes by 25%, but did not affect them in PLB-KO myocytes. Similarly, in CNP 10^{-7} M treated WT myocytes, Ca^{2+} transient peaks were reduced by 27% with only minor decreases in CNP treated PLB-KO myocytes (7%). SNAP 10^{-5} M had little effects in changing the Ca^{2+} transient peaks in either WT or PLB-KO myocytes (Figure 2).

The cyclic GMP-dependent protein phosphorylation pattern was altered in myocytes extracts from mice lacking phospholamban

Cyclic GMP-dependent protein phosphorylation reactions were performed in ventricular myocyte extracts from WT and PLB-KO mouse hearts. Protein phosphorylation patterns were compared (Figure 3). Three phosphoprotein bands at molecular masses of 170-116 kDa, a phosphoprotein of 97kDa and a band at 35kDa were significantly enhanced upon addition of cyclic GMP to proteins from WT mouse hearts (lane 2). These protein bands disappeared when KT5823, a cyclic GMP protein kinase inhibitor, was present (Figure 3, lane 3). This indicates that these proteins were targets for the cyclic GMP-dependent protein kinase. However, these phosphorylation bands were not increased (97 kDa, 35 kDa) or increased to a lesser extent (bands between 170-116 kDa) in presence of cyclic GMP in PLB-KO (Figure 3 lanes 4-6).

Fig. 3. Cyclic GMP dependent protein phosphorylation of cardiac myocytes extracts from wild type and PLB-KO mice. Lanes 1, 2, and 3 were from myocyte extracts from WT cardiac myocytes and lanes 4, 5, and 6 were from myocyte extracts from PLB-KO myocytes. Lanes 1 and 4 show baseline protein phosphorylation. The effects of cGMP/cGMP kinase are shown in lanes 2 and 5. The effects of KT 5823 are shown in lanes 3 and 6. Molecular weight standards are labeled at the left. The phosphoprotein bands that were enhanced by 8-Br-cGMP in WT myocytes are labeled on the right.

Discussion

Cyclic GMP is an important negative regulator of cardiac myocyte function. In this study, we found that 8-bromo-cGMP, CNP and SNAP all significantly decreased myocyte contractility in wild-type myocytes, while little change occurred in PLB-KO myocytes in response to these reagents. Cyclic GMP and CNP also decreased intracellular Ca^{2+} transients in WT myocytes, while these Ca^{2+} transients were unchanged in PLB-KO myocyte treated with 8-bromo-cGMP and CNP. These results demonstrated the importance of phospholamban in the functional actions of cyclic GMP on cardiac myocytes.

Cyclic GMP, the intracellular signaling molecule common to nitric oxide and natriuretic peptides, mediates an important signal transduction pathway in the cardiovascular system [16, 17]. Previous work has shown that cyclic GMP reduces myocardial metabolism, inotropy and function [1, 5]. The negative effects of cyclic GMP are mainly mediated through the cyclic GMP-dependent protein kinase. The effects of cyclic GMP may also be mediated by kinase-independent interaction, such as cyclic GMP-gated cation channels and certain phosphodiesterases [7-9]. Consistent with previous studies, our results showed that addition of the cyclic GMP

analog 8-Bromo-cGMP, CNP (a particulate guanylyl cyclase activator), and nitric donor SNAP (a soluble guanylyl cyclase activator) decreased ventricular myocytes percentage shortening, maximum rate of shortening and rate of relaxation in wild type mice. The negative effects of 8-bromo-cGMP, CNP and SNAP were blunted in PLB-KO mouse cardiac myocytes.

Particulate guanylyl cyclase and soluble guanylyl cyclase both produce cyclic GMP but are located in different intracellular sites. It has been suggested that this leads to differing effects [15]. Binding of natriuretic peptides to their receptors activates a particulate guanylyl cyclase, leading to an elevation in intracellular cyclic GMP [18, 19]. It has been shown that CNP exhibits negative inotropic effect in isolated working mouse hearts as well as rat cardiac myocytes [20, 21]. In this study, 8-bromo-cGMP and CNP decreased Ca^{2+} transients similarly to myocyte contractility in WT myocytes. This indicated that cyclic GMP produced by particulate guanylyl cyclase after stimulation with CNP might regulate myocyte contraction primarily via regulating Ca^{2+} levels.

One of the major actions of nitric oxide is to activate soluble guanylyl cyclase and increase the intracellular cyclic GMP level. Both nitric oxide and cyclic GMP have been shown to elicit negative functional and metabolic

effects in isolated cardiac myocytes as well as in the intact heart [1, 3]. In smooth muscle, it has been shown that the effects of nitric oxide are regulated through the uptake of Ca^{2+} by SERCA into intracellular stores [22]. In mouse ventricular myocytes, while SNAP significantly decreased function it had little effect on Ca^{2+} transients indicating the cyclic GMP generated by the soluble guanylyl cyclase may decrease myofilament Ca^{2+} sensitivity [15]. In the current study, intracellular Ca^{2+} transients were not affected by SNAP. The differing effects of SNAP and CNP on calcium transients suggest a differing role for natriuretic peptide generated cyclic GMP and nitric oxide generated cyclic GMP and raises the possibility of cyclic GMP compartmentalization in ventricular myocytes.

Cyclic GMP protein kinase I is a downstream target activated by the CNP- or nitric oxide-cyclic GMP signaling pathway in cardiac myocytes and this protein kinase causes phosphorylation of PLB and subsequent activation of the sarcoplasmic reticulum Ca^{2+} pump [9, 19]. Phospholamban plays a critical role in regulation of SERCA and in influencing the kinetics of calcium handling within the cardiac myocyte [14, 23]. Studies on vascular smooth muscle showed that sodium nitroprusside or cyclic GMP induced phosphorylation of phospholamban [24]. Addition of CNP or atrial natriuretic peptide to isolated cardiomyocytes increased intracellular cyclic GMP levels and also stimulated the phosphorylation of phospholamban [25]. The CNP-dependent phospholamban phosphorylation was accompanied by the activation of SERCA. In isolated mouse heart, CNP increased cardiac cyclic GMP levels and stimulated phospholamban phosphorylation. CNP and cyclic GMP also altered heart contractility suggesting that cyclic GMP induced reductions in cardiac function act through the activation of SERCA via cyclic GMP protein kinase induced phosphorylation of phospholamban [2, 5, 21]. In this study, by using PLB-KO mouse myocytes we tested the hypothesis that the negative functional effects of cyclic GMP on cardiac myocytes were mediated through cyclic GMP-dependent regulation of PLB and subsequent changes in intracellular calcium transients. We demonstrated the importance of PLB in these effects in ventricular myocytes.

PLB-KO mice have a similar phenotype compared with wild type mice. The ventricular myocytes were morphologically similar to wild type myocytes. Baseline of myocyte percent shortening of WT and PLB-KO appeared to be similar, while baseline of maximum rates of shortening and relaxation in PLB-KO were significantly higher than that in wild type mice. In vivo examination of

the PLB-KO heart has suggested increased contractility [14]. The yields of both wild type and PLB-KO were high with 50-80% of rod shaped healthy cells. The viability of the cells was confirmed by observing their contractility. Myocytes from both strains were able to contract for 20 min in the steady state (baseline level) in absence of any reagents. Functional measurements were performed on three cells per group per heart. Using these isolated ventricular myocytes, we studied the mechanisms through which cyclic GMP mediates its inhibitory effects on cardiac cell contraction.

It has been well established that there are two phosphorylation sites on phospholamban, Ser¹⁶ and Thr¹⁷ for the cyclic AMP dependent protein kinase and calcium/calmodulin-dependent protein kinase II, respectively [14]. Activation the cyclic AMP protein kinase or the calcium/calmodulin-dependent protein kinase signaling pathways leads to PLB phosphorylation and facilitates β -adrenergic receptor mediated cardiac relaxation [23]. It has also been shown that phosphorylation of PLB upon β -adrenergic receptor stimulation results in inhibition of Na^+/K^+ -ATPase activity by decreasing its $[\text{Na}]_i$ affinity [26]. Phosphorylation of PLB by the cyclic GMP-dependent protein kinase was reported in isolated sarcoplasmic reticulum from cardiac and smooth muscle [24]. Effects of nitroprusside and 8-bromo-cGMP in feline aorta were reported to be associated with an increase in Ser¹⁶ phosphorylation of PLB [27]. We have also demonstrated that cyclic GMP phosphorylates PLB in cardiac myocytes [2]. The current study demonstrates that this is a critical step in the functional actions of cyclic GMP in myocytes.

It was surprising that the cyclic GMP-dependent protein phosphorylation pattern in PLB-KO myocytes was different than that in wild type myocytes, because cyclic GMP signaling is considered as an upstream event to PLB-SERCA function. It has been reported that in smooth muscle membranes the cyclic GMP protein kinase signaling complex was associated with phospholamban and contains a number of cyclic GMP protein kinase substrates including IP_3 receptor I (IP_3RI), IRAG, and cyclic GMP protein kinase I [28-30].

This raised the possibility that the absence of PLB may cause disruption in this complex and result in changes in the protein phosphorylation pattern. Alternatively, lack of PLB may result in constant work of SERCA, which leads the accumulation of excess Ca^{2+} in the sarcoplasmic reticulum and changes in the intracellular Ca^{2+} level. It has been reported that there was higher sarcoplasmic reticulum stores of calcium in PLB-KO myocytes [31]. This might affect calcium sensitive proteins. However,

consistent with previous studies [31], our results showed that PLB-KO myocytes have similar cytosolic diastolic Ca^{2+} compared with wild type myocytes (93.3 ± 9.6 nM vs. 93.0 ± 8.3 nM). It is possible that some calcium sensitive proteins may be involved in the reduced effect of the cyclic GMP signaling pathway in PLB-KO mice.

In summary, our results demonstrated that phospholamban is required for the negative functional effects of cyclic GMP. In wild-type myocytes, 8-bromo-cGMP, CNP and SNAP reduced function and 8-bromo-cGMP and CNP reduced Ca^{++} transients. These effects were not seen in the PLB-KO myocytes. We also found

reduced cyclic GMP-dependent protein kinase activity in the PLB-KO myocytes suggesting altered functioning. Thus, the effects of cyclic GMP on cardiac myocyte function were, at least, partially mediated through the action of phospholamban in increasing Ca^{2+} re-entry to the sarcoplasmic reticulum through SERCA.

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