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Phosphorylation of contractile proteins in response to α - and β -adrenergic stimulation in neonatal cardiomyocytes

ROBERT S. DECKER, AMY K. RINES, SAKIE NAKAMURA, TEJASWITHA J. NAIK, J. ANDREW WASSERTSROM, and HOSSEIN ARDEHALI

From the Feinberg Cardiovascular Research Institute, Northwestern University Medical Center, Chicago, Ill

Abstract

α - and β -Adrenergic receptor agonists induce an inotropic response in the adult heart by promoting the phosphorylation of several regulatory proteins, including myosin-binding protein C (MyBP-C), cardiac troponin I (cTnI), and phospholamban (PLB). However, the adrenergic-induced phosphorylation of these proteins has not been characterized in the developing heart. Accordingly, we evaluated MyBP-C, cTnI, and PLB phosphorylation in cultured neonatal rat cardiomyocytes (NRCMs) after α - and β -receptor activation with phenylephrine and isoproterenol. α -Receptor stimulation increased, whereas β -receptor activation reduced MyBP-C phosphorylation. Isoelectric-focusing experiments indicated that the amount of monophosphorylated MyBP-C was sensitive to α -adrenergic activation, but diphosphorylated and triphosphorylated MyBP-C levels were largely unaffected. The phosphorylation of cTnI and PLB was consistent with the mechanism observed in adult hearts: α - and β -Receptor stimulation phosphorylated both proteins. For cTnI, the greatest difference associated with β -receptor activation was observed in the diphosphorylated state, whereas α -receptor activation was associated with a marked increase in the tetraphosphorylated protein and absence of the unphosphorylated state. Despite these apparent changes in cTnI and PLB phosphorylation, β -receptor activation failed to alter calcium transients in NRCMs. Collectively, these findings suggest that, unlike cTnI and PLB, MyBP-C and inotropy are not coupled to β -adrenergic stimulation in NRCMs. Therefore, cTnI and PLB probably play a more central role in modulating contractile function in NRCMs in response to catecholamines than does MyBP-C, and MyBP-C may have a structural role in stabilizing thick filament assembly rather than influencing cross-bridge formation in developing hearts.

The α - and β -adrenergic receptors influence cardiomyocyte contractility by modulating the phosphorylation states of contractile regulatory proteins like myosin-binding protein C (MyBP-C), cardiac troponin I (cTnI), and phospholamban (PLB).^{1–3} In adult hearts, MyBP-C phosphorylation is initiated by the stimulation of the β -adrenergic receptor, which leads to the addition of phosphate groups at 3 N-terminal serine (Ser) residues.^{4–9} β -receptor stimulation also induces the phosphorylation of PLB, and both α - and β -receptor activation induce cTnI phosphorylation. MyBP-C phosphorylation is believed to alter the stability and packing of myosin molecules in the thick filament^{10,11} and to allow the myosin heads to interact freely with the thin filament, thereby accelerating cross-bridge formation and increasing the contractile force.^{1,10,11} cTnI phosphorylation modulates contractility by altering the sensitivity of the contractile apparatus to calcium during contraction,¹² whereas PLB phosphorylation regulates Ca^{2+} availability for contraction.^{13,14}

The adrenergic-induced phosphorylation of MyBP-C, cTnI, and PLB has not been characterized in the developing heart, so we examined this process with a series of experiments performed in neonatal rat cardiomyocytes (NRCMs). The phosphorylation of cTnI and PLB was largely consistent with the mechanism observed in adult hearts: β -Receptor stimulation phosphorylated both proteins, and α -receptor stimulation reduced the amount of unphosphorylated cTnI. However, the influence of adrenergic-receptor activation on MyBP-C phosphorylation differed substantially. α -Receptor stimulation increased the amount of phosphorylated MyBP-C in NRCMs, and β -receptor activation reduced, rather than enhanced, MyBP-C phosphorylation. β -receptor activation also failed to alter calcium transients in NRCMs and, consequently, may not lead to NRCM contraction. Collectively, our findings suggest that MyBP-C is not essential for the regulation of contractility in neonatal cardiomyocytes and seems to be uncoupled from the β -adrenergic receptor unlike PBL and cTnI.

MATERIALS AND METHODS

NRCM isolation and treatment

NRCMs were isolated from the hearts of 2- and 3-day-old rats. Hearts were harvested, diced in Ca^{2+} -free Hanks' solution, and incubated in trypsin, then the isolated cardiomyocytes were partially purified via differential adhesion, plated on 35-mm plates (1.2×10^6 cells/plate), and cultured for 1 week at 37°C in Dulbecco's modified eagle medium (DMEM) containing 5% fetal calf serum and vitamin B12 as described previously.¹⁵ Experimental treatments consisted of phenylephrine (1 $\mu\text{mol/L}$), isoproterenol (iso) (1 $\mu\text{mol/L}$), prazosin (10 $\mu\text{mol/L}$), propranolol (10 $\mu\text{mol/L}$), KN-93 (2 $\mu\text{mol/L}$), and forskolin (10 $\mu\text{mol/L}$) alone and in the combinations indicated. Dose responses were undertaken with adrenergic agonists and specific blockers and/or inhibitors in the NRCM cultures. The cells were treated in serum-free medium for 24 h and then rinsed in Hanks' solution before subsequent analyses were performed. All protocols were approved by the Institutional Animal Care and Use Committee of Northwestern University.

MyBP-C, cTnI, and PLB phosphorylation

Regulatory protein levels were assessed via Western blots. The total MyBP-C content was assessed with a polyclonal antibody that we developed against the N-terminus of MyBP-C but does not distinguish among the phosphorylation states; phosphorylated MyBP-C levels were assessed with an antibody that specifically recognizes phosphorylated Ser at amino acid position 282.^{9,16} The total cTnI content was assessed with an antibody that recognizes the protein (University of Iowa Hybridoma Collection). The phosphorylated cTnI levels were assessed with an antibody that recognizes phosphorylated Ser23/24 (CalBiochem, San Diego, Calif). PLB phosphorylation was assessed with an antibody that recognizes phosphorylated Ser16 (Signal Transduction, Boston, Mass). Protein levels were determined via densitometry and were normalized to either total MyBP-C or actin levels.

Separation of phosphorylation states

The phosphorylation state of MyBP-C was monitored with 1-dimensional isoelectric focusing,^{9,14} and cTnI was separated via the nonequilibrium isoelectric focusing protocol developed by Kobayashi et al.¹⁷ Approximately 5 μg of cell extract was loaded on 1-dimensional isoelectric-focusing slab gels. MyBP-C phosphorylation states were separated between pH 3 and pH 7, and cTnI phosphorylation states were separated between pH 7 and pH 9. PLB phosphorylation states were separated by isolating PLB monomers and pentamers.

MyBP-C cellular distribution

Cultured myocytes were fixed in 4% formaldehyde for 5 min, rinsed in phosphate-buffered saline, and extracted in 0.2% Triton X-100, then stained with the antibody that recognizes the N-terminus of MyBP-C or with the antibody that recognizes phosphorylated Ser282.^{9,16} MyBP-C antibodies were visualized via secondary staining with FITC-conjugated goat anti-rabbit antibodies or rhodamine-conjugated goat anti-rabbit immunoglobulin G1, and the cells were viewed with a Zeiss 510 laser-scanning confocal microscope (Carl Zeiss IMT Corporation, Maple Grove, Minn).¹⁶ The fluorescence (F) intensity was determined from digital images obtained with Zeiss 510 software.

Calcium-transient measurements

NRCMs were attached to 18-mm coverslips and loaded with 15 $\mu\text{mol/L}$ fluo4 AM for 20 min, then washed in serum-free DMEM, placed in an experimental chamber adapted for temperature control and electrical stimulation (Cell MicroControls, Norfolk, Va), and perfused with DMEM at 35°C. The spontaneous beating rates of the NRCMs were measured. The cells were then paced at 2 Hz with a square pulse generator set at a stimulation intensity of 10 V and duration of 10 ms. Iso (1–10 $\mu\text{mol/L}$) was applied to individual clusters of beating myocytes, and Ca^{2+} levels were monitored by measuring fluo4 AM F (excitation wavelength: 483 nm, emission wavelength: 512 nm) with a Zeiss 510 laser scanning confocal microscope. The ratio of peak F to resting F₀ was calculated by measuring F intensity in myocyte clusters. Calcium transients (F/F₀) were recorded, and the transient amplitude was calculated for 8 myocyte clusters.

Statistical analysis

Data are expressed as mean $\pm$ standard error of measurement. Statistical significance was assessed with the unpaired Student *t*-test; a *P* value of less than 0.05 was considered statistically significant.

RESULTS

MyBP-C phosphorylation

Adrenergic-induced phosphorylation was assessed in NRCMs treated with and without phenylephrine (an α -receptor agonist) or iso (a β -receptor agonist). Monophosphorylated MyBP-C (MyBP-C-P₁) was identified with an antibody to phosphorylated Ser282 (anti-Ser282-P). Western blot analyses indicated that MyBP-C-P₁ levels were nearly 50% higher ($P < 0.05$) in NRCMs treated with phenylephrine and approximately 45% lower in NRCMs treated with iso ($P < 0.05$) (Fig 1, A–D) than in untreated cells. Furthermore, treatment with the α -receptor blocker prazosin significantly reduced MyBP-C-P₁ levels ($P < 0.05$) without affecting the diphosphorylated and triphosphorylated forms of MyBP-C (MyBP-C P_{2,3}) and abolished the enhancement induced by phenylephrine. These observations suggest that in neonatal cardiomyocytes, MyBP-C phosphorylation is increased by α -adrenergic activation and decreased by β -adrenergic activation.

Cellular MyBP-C can exist in any of 4 phosphorylation states: unphosphorylated, monophosphorylated (MyBP-C-P₁), diphosphorylated (MyBP-C-P₂), or triphosphorylated (MyBP-C-P₃). To determine whether the adrenergic stimulation of NRCMs alters the distribution of phosphorylated forms of MyBP-C, the NRCMs were treated with and without phenylephrine, phenylephrine and prazosin, or iso, and then the phosphorylation states were separated by 1-dimensional isoelectric focusing and stained with antibodies that bind to the N-terminal MyBP-C domain (Fig 2). The total MyBPC levels in treated and untreated cells were similar, and the 3 phosphorylated states were identified. The unphosphorylated form of MyBP-C could not be detected in these experiments. MyBP-CP₁ levels were 2-fold greater

in cells treated with phenylephrine than in untreated cells but markedly declined with iso treatment; neither treatment altered MyBP-C-P₂ or MyBP-C-P₃ levels. Cotreatment with prazosin abolished the phenylephrine-induced increase in MyBP-C-P₁, whereas the levels of MyBP-C-P₂ and MyBP-C-P₃ declined insignificantly (Fig 2). Thus, the amount of MyBP-C-P₁ in NRCMs is sensitive to adrenergic activation, but the levels of MyBP-C-P₂ and MyBP-C-P₃ are largely unaffected.

The cellular location of MyBP-C in NRCMs was assessed by fluorescently staining cultured cells with the N-terminal MyBP-C antibody or with the anti-Ser282-P antibody. In the absence of adrenergic-receptor activation, MyBP-C was present in the C-zone of the A-band of nascent myofibrils (Fig 3, A). After adrenergic activation, the proportion of myofibril-localized MyBP-C-P₁₋₃ was higher in cells treated with phenylephrine than in iso-treated cells, and the amount of MyBP-C-P₁₋₃ was nearly 2-fold greater after treatment with phenylephrine than after iso treatment (Fig 3, B and C) ($P < 0.01$). The distribution of the Ser 282 epitope of MyBP-C in response to phenylephrine or iso exposure was different. The Ser 282 site was observed in filaments (arrows, Fig 3, B) and myocyte cytoplasm after phenylephrine treatment. In contrast, iso reduced Ser 282 staining and the epitope was diffusely distributed in the myocytes not associated with myofibrils.

We then conducted experiments to study the mechanism for the decrease in MyBP-C phosphorylation in NRCMs. In adult hearts, MyBP-C Ser282 is phosphorylated by Cam-kinase II, and subsequent phosphorylation steps are catalyzed by protein kinase A (PKA).^{4,5,9} We investigated whether these enzymes contribute to MyBP-C phosphorylation in NRCMs. The MyBP-C-P₁₋₃ levels were similar in untreated NRCMs, in NRCMs treated with the Cam-kinase II inhibitor KN-93, and in NRCMs treated with both KN-93 and iso (Fig 4). However, treatment with forskolin, which upregulates PKA expression by inducing cyclic AMP (cAMP), significantly increased MyBP-C-P₁₋₃ levels ($P < 0.05$). Collectively, these results suggest that PKA contributes to MyBP-C phosphorylation in NRCMs, and the defects in communication between this protein and β -adrenergic receptor may explain the lack of MyBP-C phosphorylation in NRCMs.

cTnI and PLB phosphorylation

NRCM extracts were examined to assess total cTnI levels or its phosphorylated form (cTnI-P) with antibodies to phosphorylated Ser23/24 (anti-Ser23/24-P). The total cTnI levels in untreated NRCMs and in NRCMs treated for 24 h with iso, iso and propranolol (a β -receptor blocker), or phenylephrine were similar. However, the level of cTnI-P was $69.4\% \pm 5.3\%$ ($P < 0.01$) higher in NRCMs treated with iso than in untreated cells, and cotreatment with propranolol abolished this enhancement (Fig 5, A). When the phosphorylation states were separated via nonequilibrium iso-electric focusing, the greatest difference was associated with isoproterenol treatment, which increased the diphosphorylated form of cTnI ($72\% \pm 13\%$, $P < 0.05$, $n = 4$) (Fig 5, B). Phenylephrine did not alter cTnI-P₂ levels, but the amount of tetraphosphorylated cTnI was markedly higher, and the unphosphorylated protein was nearly absent. Thus, β -receptor stimulation increased cTnI diphosphorylation, and α -receptor stimulation increased the relative amount of tetraphosphorylated cTnI.

The total and phosphorylated PLB levels were assessed with antibodies to total and to phosphorylated Ser16 (anti-Ser16-P) of PLB, respectively. Treatment with phenylephrine or isoproterenol did not alter total PLB levels (data not shown). The amounts of phosphorylated PLB in untreated cells and in cells treated with phenylephrine were similar; however, treatment with isoproterenol increased the levels of phosphorylated PLB monomers and pentamers (Fig 6). Thus, PLB phosphorylation increased when the β -receptor was stimulated but not after α -receptor stimulation. Furthermore, because the phosphorylation states of PLB monomers and pentamers differ, β -receptor stimulation

increases total PBL phosphorylation and seems to increase the phosphorylation of the pentamer to a greater degree than the monomer.

Effect of isoproterenol on Ca^{2+} transients in NRCMs

Because PLB and cTnI regulate cardiomyocyte contractility by modulating cytoplasmic free- Ca^{2+} levels concentration^{13,14} and the Ca^{2+} sensitivity of the contractile apparatus,¹² respectively, we assessed the potential impact of β -receptor activation on NRCM function by recording intracellular Ca^{2+} transients in cells treated with and without iso. NRCMs were paced at a frequency of 2 Hz, and the transients were monitored with the Ca^{2+} fluorescent marker fluo4 AM. Treatment with a relatively high concentration (1–10 $\mu\text{mol/L}$) of iso did not alter the shape or the amplitude of the Ca^{2+} transients (Fig 7); thus, β -activation does not induce an inotropic response in NRCMs, despite the apparent increase in PLB and cTnI phosphorylation.

DISCUSSION

The phosphorylation of contractile regulatory proteins in adult cardiac tissue has been described previously,^{1,2} but the corresponding mechanisms in the developing heart are characterized poorly. Here, we investigated the contribution of α - and β -adrenergic stimulation to the phosphorylation of MyBP-C, cTnI, and PLB in neonatal cardiomyocytes. Our results indicate that cTnI and PLB phosphorylation in NRCMs seems similar to the mechanism observed in adult hearts: α -Receptor stimulation reduced the amount of unphosphorylated cTnI, and β -receptor stimulation phosphorylated both proteins. However, we observed substantial differences in the mechanism of MyBP-C phosphorylation. α -Stimulation doubled the amount of monophosphorylated MyBP-C in NRCMs with no concurrent changes in diphosphorylated and triphosphorylated MyBP-C levels, whereas β -receptor stimulation reduced, rather than increased, MyBP-C phosphorylation. β -Receptor stimulation also failed to convert monophosphorylated MyBP-C to either the diphosphorylated or triphosphorylated forms, as observed in adult cardiomyocytes,^{4,5,9,18} and it did not change the shape or amplitude of Ca^{2+} transients in NRCMs. Collectively, these findings suggest that α -adrenergic stimulation may play a minor role in MyBP-C-mediated contractile regulation¹⁹ and that the coupling of β -receptor activation to MyBP-C phosphorylation and the regulation of contractility is limited in cultured NRCMs or develops at a later stage of maturation. β -receptor-induced MyBP-C phosphorylation seems to be essential for the proper regulation of cardiac force generation in adult hearts.^{9,11,19} In mice, the expression of constitutively phosphorylated MyBP-C increased contractility,¹⁸ and a MyBP-C knockout mutation^{20,21} or mutations that prevent MyBP-C phosphorylation¹⁸ induced contractile abnormalities that did not improve with β -receptor stimulation.²² However, after stimulating the β -receptor in NRCMs, we found most of the phosphorylated MyBP-C in the cytosol, whereas α -receptor stimulation led to the accumulation of phosphorylated MyBP-C in myofibrils. Furthermore, α -adrenergic stimulation of protein kinase C has been linked to MyBP-C phosphorylation *in vitro* and cardiomyocyte growth.²³ Thus, MyBP-C phosphorylation mediated by the α -receptor may be related to myofibril assembly and structure,^{11,24} whereas the β -receptor seems to have little or no role in neonatal cardiomyocytes. The phosphorylation of MyBP-C is believed to occur in 2 steps in adult cardiomyocytes: First, Cam-kinase II catalyzes the addition of a phosphate group at Ser282, followed by PKA phosphorylation of other Ser residues mediated by β -adrenergic activation.^{4,5,9,18,19} However, the phosphorylated MyBP-C levels in NRCMs were not altered by Cam-kinase II inhibition, and MyBP-C phosphorylation is increased in response to cAMP-induced PKA upregulation by forskolin but not iso. Thus, in neonatal cells, the mechanism of MyBP-C phosphorylation has yet to mature (ie, coupled to the β -receptor) to the “adult” state.¹⁹

Unlike MyBP-C, which is believed to regulate contractility by loosening the packing of myosin tails in the thick filament^{11,16,24} and by promoting interactions between the myosin heads and the actin thin filaments,^{8,9,11,24} cTnI and PLB regulate contractility by influencing the sensitivity of the contractile apparatus to calcium¹² and by modulating the intracellular concentration of Ca²⁺.^{13,14} Our studies indicate that the β -receptor-induced phosphorylation of cTnI and PLB observed in adult hearts also occurs in neonatal cells, but it was not accompanied by changes in the shape or amplitude of the calcium transients. Our results are different from those of Wu et al, who found an increase in Ca²⁺ transients in NRCMs in response to iso,²⁵ which could be caused by differences in the treatment duration and conditions. Wu et al treated NRCMs with iso for 48–72 h, compared with overnight treatment (ie, 12 h) in our experiments.²⁵ Furthermore, we treated NRCMs with iso under serum-free conditions, whereas Wu et al maintained serum in their culture media, which can contribute to NRCM differentiation and growth. However, the question may arise as to why the Ca²⁺ transients did not change in our model despite an increase in cTnI and PLB phosphorylation. We propose that in our model, the iso-induced phosphorylation of PBL or cTnI is not coupled with positive inotropy as evidenced by the lack of changes in the amplitude of the calcium transient. Perhaps these results reflect the relative undifferentiated state of the sarcoplasmic reticulum, the lack of close associations between developing T-tubules and the junctional sarcoplasmic reticulum, and the immature state of the myofibril in NRCMs.^{1,20} These observations suggest that the responsiveness of contractile regulatory proteins to iso may develop at different rates in neonatal cardiomyocytes.

In summary, the adrenergic-receptor-induced phosphorylation of cTnI and PLB was largely consistent with the mechanisms observed in adult hearts; however, we observed substantial differences in the mechanisms associated with MyBP-C phosphorylation. the activation of the α -receptor increased the amount of phosphorylated MyBP-C in NRCMs, and β -receptor activation reduced, rather than increased, phosphorylated MyBP-C levels. β -Receptor activation also failed to alter calcium transients in NRCMs and, consequently, may not induce an inotropic response. Because α adrenergic agonists have been demonstrated to stimulate neonatal myocyte growth and myofibril assembly, perhaps phosphorylation of MyBP-C by phenylephrine is related to the stabilization of these nascent myofibrils in the rapidly growing heart cells. Our findings suggest collectively that the coupling of β -receptor activation to MyBP-C phosphorylation and the regulation of contractility is limited in young, cultured NRCMs and may occur at a later stage of development when the regulation of contractile performance is of paramount importance.

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Abbreviations

cAMP	cyclic AMP
cTnI	cardiac troponin I
DMEM	Dulbecco's modified eagle medium
F	fluorescence
iso	isoproterenol
MyBP-C	myosin binding protein-C
MyBP-C-P1	monophosphorylated MyBP-C

RCM	neonatal rat cardiomyocyte
PKA	protein kinase A
PLB	phospholamban
Ser	serine

REFERENCES

1. Gordon AM, Homsher E, Regnier M. Regulation of contraction in striated muscle. *Physiol Rev.* 2000; 80:853–924. [PubMed: 10747208]
2. Solaro RJ, Montgomery DM, Wang L, et al. Integration of pathways that signal cardiac growth with modulation of myofilament activity. *J Nucl Cardiol.* 2002; 9:523–33. [PubMed: 12360133]
3. Solaro RJ, Rarick HM. Troponin and tropomyosin: proteins that switch on and tune in the activity of cardiac myofilaments. *Circ Res.* 1998; 83:471–80. [PubMed: 9734469]
4. Gautel M, Zuffardi O, Freiburg A, Labeit S. Phosphorylation switches specific for the cardiac isoform of myosin binding protein-C: a modulator of cardiac contraction? *EMBO J.* 1995; 14:1952–60. [PubMed: 7744002]
5. Gruen M, Prinz H, Gautel M. cAPK-phosphorylation controls the interaction of the regulatory domain of cardiac myosin binding protein C with myosin-S2 in an on-off fashion. *FEBS Lett.* 1999; 453:254–9. [PubMed: 10405155]
6. Hartzell HC. Effects of phosphorylated and unphosphorylated C-protein on cardiac actomyosin ATPase. *J Mol Biol.* 1985; 186:185–95. [PubMed: 2934553]
7. Kulikovskaya I, McClellan G, Flavigny J, Carrier L, Winegrad S. Effect of MyBP-C binding to actin on contractility in heart muscle. *J Gen Physiol.* 2003; 122:761–74. [PubMed: 14638934]
8. Kunst G, Kress KR, Gruen M, Uttenweiler D, Gautel M, Fink RH. Myosin binding protein C, a phosphorylation-dependent force regulator in muscle that controls the attachment of myosin heads by its interaction with myosin S2. *Circ Res.* 2000; 86:51–8. [PubMed: 10625305]
9. McClellan G, Kulikovskaya I, Winegrad S. Changes in cardiac contractility related to calcium-mediated changes in phosphorylation of myosin-binding protein C. *Biophys J.* 2001; 81:1083–92. [PubMed: 11463649]
10. Kulikovskaya I, McClellan GB, Levine R, Winegrad S. Multiple forms of cardiac myosin-binding protein C exist and can regulate thick filament stability. *J Gen Physiol.* 2007; 129:419–28. [PubMed: 17470661]
11. Levine R, Weisberg A, Kulikovskaya I, McClellan G, Winegrad S. Multiple structures of thick filaments in resting cardiac muscle and their influence on cross-bridge interactions. *Biophys J.* 2001; 81:1070–82. [PubMed: 11463648]
12. Sumandea MP, Burkart EM, Kobayashi T, De Tombe PP, Solaro RJ. Molecular and integrated biology of thin filament protein phosphorylation in heart muscle. *Ann NY Acad Sci.* 2004; 1015:39–52. [PubMed: 15201148]
13. Kim SJ, Depre C, Vatner SF. Novel mechanisms mediating stunned myocardium. *Heart Fail Rev.* 2003; 8:143–53. [PubMed: 12766493]
14. Kim SJ, Kudej RK, Yatani A, et al. A novel mechanism for myocardial stunning involving impaired Ca(2+) handling. *Circ Res.* 2001; 89:831–7. [PubMed: 11679414]
15. Ardehali H, O'Rourke B, Marban E. Cardioprotective role of the mitochondrial ATP-binding cassette protein 1. *Circ Res.* 2005; 97:740–2. [PubMed: 16166555]
16. Decker RS, Decker ML, Kulikovskaya I, et al. Myosin-binding protein C phosphorylation, myofibril structure, and contractile function during low-flow ischemia. *Circulation.* 2005; 111:906–12. [PubMed: 15699252]
17. Kobayashi T, Yang X, Walker LA, Van Breemen RB, Solaro RJ. A non-equilibrium isoelectric focusing method to determine states of phosphorylation of cardiac troponin I: identification of Ser-23 and Ser-24 as significant sites of phosphorylation by protein kinase C. *J Mol Cell Cardiol.* 2005; 38:213–8. [PubMed: 15623438]

18. Sadayappan S, Gulick J, Osinska H, et al. Cardiac myosin-binding protein-C phosphorylation and cardiac function. *Circ Res.* 2005; 97:1156–63. [PubMed: 16224063]
19. Flashman E, Redwood C, Moolman-Smook J, Watkins H. Cardiac myosin binding protein C: its role in physiology and disease. *Circ Res.* 2004; 94:1279–89. [PubMed: 15166115]
20. Carrier L, Knoll R, Vignier N, et al. Asymmetric septal hypertrophy in heterozygous cMyBP-C null mice. *Cardiovasc Res.* 2004; 63:293–304. [PubMed: 15249187]
21. Harris SP, Bartley CR, Hacker TA, et al. Hypertrophic cardiomyopathy in cardiac myosin binding protein-C knockout mice. *Circ Res.* 2002; 90:594–601. [PubMed: 11909824]
22. Herron TJ, Rostkova E, Kunst G, Chaturvedi R, Gautel M, Kentish JC. Activation of myocardial contraction by the N-terminal domains of myosin binding protein-C. *Circ Res.* 2006; 98:1290–8. [PubMed: 16614305]
23. Mohamed AS, Dignam JD, Schlender KK. Cardiac myosin-binding protein C (MyBP-C): identification of protein kinase A and protein kinase C phosphorylation sites. *Arch Biochem Biophys.* 1998; 358:313–9. [PubMed: 9784245]
24. Weisberg A, Winegrad S. Alteration of myosin cross bridges by phosphorylation of myosin-binding protein C in cardiac muscle. *Proc Natl Acad Sci U S A.* 1996; 93:8999–9003. [PubMed: 8799143]
25. Wu G, Wang H, Yang J, Liu C, Jing L, et al. Kappa-opioid receptor stimulation inhibits augmentation of Ca(2+) transient and hypertrophy induced by isoprenaline in neonatal rat ventricular myocytes—Role of CaMKIIdelta(B). *Eur J Pharmacol.* 2008; 595:52–7. [PubMed: 18721804]

AT A GLANCE COMMENTARY**Background**

The activities of myosin binding protein-C (MyBP-C), cardiac troponin I (cTnI), and phospholamban are regulated by phosphorylation in adult cardiomyocytes. The phosphorylation of MyBP-C accelerates cross-bridge formation; cTnI phosphorylation alters calcium sensitivity of the contractile apparatus, and phospholamban phosphorylation regulates intracellular $[Ca^{2+}]$. β -Adrenergic stimulation mediates the phosphorylation of MyBP-C, cTnI, and PLB, whereas α agonists only phosphorylate cTnI. It is unclear how adrenergic stimulations regulate the phosphorylation of these proteins during cardiac development.

Translational Significance

β -Adrenergic stimulation decreases MyBP-C phosphorylation in neonatal cells, whereas cTnI and PLB phosphorylation are both increased with β -stimulation. These results suggest that, unlike adult hearts, β -adrenergic stimulation in developing hearts may have cardiac inhibitory effects. Thus, neonatal heart may respond differently to phosphodiesterase inhibitors and inotropes than adult hearts.

Fig 1.

Adrenergic-receptor-induced MyBP-C phosphorylation in NRCMs. NRCMs were treated with and without (**A, B**) phenylephrine, prazosin, phenylephrine and prazosin, or (**C, D**) iso. Then, the phosphorylated MyBP-C (MyBP-C-P₁₋₃) levels were assessed via Western blots with an antibody to phosphorylated Ser282 and normalized to the total MyBP-C levels. * $P < 0.05$ versus untreated; $n \geq 6$ per treatment group.

Fig 2.

Phosphorylation states of MyBP-C in NRCMs after adrenergic-receptor activation. NRCMs were treated with and without phenylephrine, phenylephrine and prazosin, or iso; then, the phosphorylation states were separated via isoelectric focusing and stained with antibodies that bind to the N-terminal MyBP-C domain.

Fig 3. Cellular distribution of MyBP-C in NRCM. NRCM were cultured (A) in the absence of adrenergic-receptor activation and after treatment with (B) phenylephrine or (C) iso. The total MyBP-C expression was identified by staining with antibodies that bind to the N-terminal MyBP-C domain (A), and phosphorylated MyBP-C (MyBP-C1P₁₋₃) was identified with antibodies to phosphorylated Ser282 (B, C). (Color version of figure is available online.)

Fig 4.

MyBP-C phosphorylation in NRCM: the roles of Cam-kinase II and PKA. NRCM were treated with and without KN-93, KN93 and iso, or forskolin, then phosphorylated MyBP-C (MyBP-C-P₁₋₃) levels were assessed with an antibody to phosphorylated Ser282 and normalized to the level observed in untreated cells. * $P < 0.05$ versus untreated; $n \geq 3$ per treatment group.

Fig 5.

Adrenergic-receptor-induced cTnI phosphorylation in NRCMs. NRCMs were treated with and without iso, iso and propanolol, or phenylephrine. **(A)** The total and phosphorylated cTnI levels were assessed via Western blots; the total cTnI was identified with antibodies that bind to the total cTnI, and the phosphorylated cTnI (cTnI-P) was identified with antibodies to phosphorylated Ser23/24. **(B)** The phosphorylation states were separated via isoelectric focusing.

Fig 6.

Adrenergic-receptor–induced PLB phosphorylation in NRCMs. NRCMs were treated with and without iso or phenylephrine, then PLB monomers and pentamers were separated, and the phosphorylated PLB levels were assessed with antibodies to phosphorylated Ser16. (Color version of figure is available online.)

Fig 7.

Effect of iso on Ca^{2+} transients in NRCM. Cultured NRCM were loaded with the Ca^{2+} fluorescent marker fluo4 AM, and the beat rate was paced at 2 Hz; then NRCM clusters were treated with or without iso. The Ca^{2+} transients were monitored via fluo4 AM F. (A) The calcium transients were recorded as the ratio of beat-stimulated (F) and ambient (F_0) F, and (B) transient amplitudes in treated and untreated cells were calculated for 8 separate NRCM clusters.