Research Article

Synergistic Effects between Phosphorylation of Phospholamban and Troponin I Promote Relaxation at Higher Heart Rate

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We hypothesized that the extent of frequency-dependent acceleration of relaxation (FDAR) would be less than that of isoproterenol-(ISO-)dependent acceleration of relaxation (IDAR) at the same increment of heart rates, and ISO may improve FDAR. Cardiac function and phosphorylation of PLB and cTnI were compared in pacing, ISO treatment, and combined pacing and ISO treatment in isolated working heart. The increase in cardiac output and the degree of relaxation was less in pacing than in ISO treatment at the same increment of heart rates. The increasing stimulation frequency induced more significant relaxant effect in ISO perfusion than that in physiological salt perfusion. The pacing only phosphorylated PLB at Thr17, but ISO induced phosphorylation of cTnI and PLB at Ser16 and Thr17. Those results suggest that the synergistic effects of PLB and cTnI induce higher degree of relaxation which makes a sufficient diastolic filling of the ventricle at higher heart rate.

1. Introduction

Heart rate is a fundamental physiological modulator of cardiac function in all mammalian hearts. Regardless of whether the force-frequency relationship is positive or negative, an increase in heart rate is always associated with an acceleration of relaxation, named as frequency-dependent acceleration of relaxation (FDAR) [1]. This FDAR ensures the proper relaxation and diastolic filling of the ventricle at higher heart rates. Previous studies indicate that Ca^{2+}/Calmodulin-dependent kinase II (CaMKII) is a prime candidate in sensing and mediating frequency-dependent effects [2]. The CaMKII has at least four substrates involved in excitation-contraction coupling in the heart [3]. Published studies demonstrate that CaMKII can phosphorylate ryanodine receptor (RyR) [4], sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) [5], phospholamban (PLB) [6], and the L-type Ca^{2+} channels (LTCC) [7]. The phosphorylation of LTCC and RyR is related to modulation to systolic function. It has been reported that LTCC activation by CaMKII is involved in the development of early afterdepolarizations and arrhythmias in vitro and in vivo [8]. Huke and Bers found that FDAR did not rely

on phosphorylation of RyR [1]. Valverde et al. reported that SERCA phosphorylation by CaMKII was not involved in FDAR [9]. Thus, a very likely target of FDAR is PLB, an inhibitor of SERCA [10]. When PLB becomes phosphorylated at Thr-17 by CaMKII, the inhibition is relieved, and Ca^{2+} uptake of SR is accelerated [10]. However, increasing stimulation frequency still induces FDAR in PLB knockout mouse hearts [11]. Therefore, the molecular mechanism of FDAR is still controversial.

Activation of the sympathetic nervous system leads to the secretion of catecholamines, which stimulate β-adrenergic receptors in the heart. Isoproterenol-(ISO-)dependent acceleration of relaxation (IDAR) is another important intrinsic regulator to cardiac function. Stimulation of β-adrenergic receptor (β-AR) by ISO activates Gs, which in turn activates adenylate cyclase and increases the formation of cAMP within cardiomyocytes. Elevated levels of cAMP increases the activation of protein kinase A (PKA) which phosphorylates intracellular targets such as LTCC, RyR, PLB, cardiac troponin I (cTnI), and myosin binding protein C (MyBPC). Phosphorylation of LTCCs enhances Ca^{2+} influx which increases shortening amplitude or force of contraction

[7]. Recently, MacDonnell et al. reported that adrenergic regulation of cardiac contractility did not involve phosphorylation of the cardiac RyR at serine-2808 residue [12]. Phosphorylation of MyBPC does not appear to have any effect on myofibrillar Ca^{2+} sensitivity which modulates relaxation but accelerated the kinetics of force development [13]. Phosphorylated MyBPC may link to myocardial protection in ischemia [14, 15]. The elevated intracellular Ca^{2+} concentration increases the activity of SERCA which indirectly accelerates relaxation of cardiomyocytes. The phosphorylated PLB at Ser-16 and Thr-17 by PKA also enhanced the activity of SERCA. The phosphorylation of cTnI reduced the Ca^{2+} sensitivity of myofibrils which facilitates Ca^{2+} dissociation from myofibrils [16]. Therefore, phosphorylation of PLB and cTnI may be two synergistic modulators to accelerate relaxation in ISO stimulation.

The increasing frequency of stimulation only phosphorylates one site of PLB, but not cTnI. Due to lack of the synergistic effect between PLB and cTnI, we hypothesized that the extent of FDAR would be less than that of IDAR at the same increment of heart rate, the less extent of FDAR may have a limitation on the sufficient diastolic filling of the ventricle with increasing frequency of stimulation, and ISO may improve FDAR. Therefore, the aim of the present study was to compare the cardiac function and extent of relaxation between FDAR and IDAR at the same increment of heart rate and further to observe the extent of FDAR during ISO stimulation.

2. Materials and Methods

2.1. Preparation of Isolated Working Heart. Sprague-Dawley rats weighing 220 ± 10 g were used. The animal procedure described here has been approved by the Animal Care and Use Committee at the Fourth Military Medical University.

The isolated crystalloid-perfused rat heart has previously been described [17]. Rats were heparinized (500 U i.p.) and anesthetized (sodium pentobarbital, 40 mg/kg i.p.). Hearts were excised, immediately mounted, and perfused on a Langendorff apparatus (Radnoti, USA) at a constant pressure of 60 mmHg with Krebs-Henseleit solution (in mM: 118 NaCl, 4.7 KCl, 2.25 $MgSO_4$, 2.25 $CaCl_2$, 23.8 $NaCO_3$, 1.2 NaH_2PO_4, 0.32 EDTA, and 11.5 D-glucose) in a nonrecirculating retrograde mode. The time from excision to perfusion was less than 60 seconds. The perfusate was oxygenated with a gas mixture of 95% O_2-5% CO_2, achieving pH of 7.39 to 7.41. Left atrium was cannulated through the pulmonary vein with steel cannula (inside diameter: 1.8 mm; outside diameter: 2.0 mm). In order to detect the intraventricular pressure, an ultraminiature pressure catheter transducer (model SPR-671; Millar Instruments, Houston, USA) was put into the left ventricle through the left atrium. The preload was set up at 10 mmHg. The heart was then switched from the Langendorff to a working mode. The aortic and coronary flow was recorded at the 1st, 5th, 10th, and 15th min at the working heart mode. Electrodes placed on the aortic cannula and the apex of the ventricle were connected to a square wave stimulator (Aurora Scientific Inc., Canada, 5 ms pulse duration, 20 V pulse amplitude). Hearts were paced at

300, 360, and 420 beats/min in the absence and the presence of isoproterenol (ISO, Sigma Chemical Co.). The hearts were superfused with 1.0, 10.0, and 20.0 nM of ISO for at least 5 min. The aortic flow, coronary flow, heart rate, left ventricular end-systolic (LVESP) and end-diastolic (LVEDP) pressure, and maximal rates of left ventricular pressure development ($+dP/dt_{max}$) and relaxation ($-dP/dt_{max}$) were measured to evaluate the contractile function of isolated working heart. Cardiac output is equal to aortic flow plus coronary flow. Data was acquired and analyzed using a PowerLab system and Chart software (ADInstruments Inc., Sydney, Australia).

2.2. Intracellular Ca^{2+} Measurement of the Single Cardiomyocyte. The ventricular myocyte isolation was the same as that described previously [18]. The cannulated hearts were mounted on a Langendorff perfusion apparatus and perfused with Ca^{2+}-free Joklik's modified minimum essential medium (Sigma-Aldrich, St. Louis, Mo, USA) containing 10 mM HEPES, 0.1% bovine serum albumin (BSA). After 5 min, the perfusate was switched to a circulating enzyme solution containing 0.08% collagenase I (Sigma-Aldrich) for 30 min. All the perfusion procedure was performed at 37°C in a constant flow, and the perfusion pressure was monitored. Then the ventricular tissues were chopped, and the cardiomyocytes were dispersed gently by a wide tipped pipette. The cell suspension was filtered through 200-μm nylon mesh. After 30 min, the cells were resuspended in Joklik's medium containing 1% BSA and slowly recovered Ca^{2+} concentration to 1.25 mM.

The intracellular Ca^{2+} concentration of cardiomyocytes was measured within 6 hours after isolation. Ventricular myocytes were loaded with fura-2 AM (5 μM) for 30 min in the dark at room temperature. Cells were then transferred into a 0.2 mL chamber which was on the stage of an inverted microscope (OlympusX71, Tokyo, Japan). Cells were superfused with Tyrode solution (in mM: 132 NaCl, 4.8 KCl, 1.2 $MgCl_2$, 1.8 $CaCl_2$, 5.0 sodium pyruvate, 10 HEPES, and 10 D-glucose, pH 7.40) at a flow rate of 0.2 mL/min at 37°C. Electric field stimulus (rectangle wave, 15 V, 5 ms, 1.0 Hz) was given by the stimulator. Then the stimulation frequency increased to 2 Hz, 5 Hz, and 10 Hz with or without ISO perfusion (1, 10, and 20 nM).

Intracellular Ca^{2+} concentration was measured by whole cell photometry (DeltaRam, Photon Technology International, USA). The emission ratio at 510 nm, during alternate excitation at 340 nm and 380 nm was used to determine intracellular Ca^{2+} concentration. Fluorescence emission was measured at 240 points/s for each of the excitation wavelengths. Background fluorescence values were determined at each excitation wavelength. These background values were subtracted from the recordings made at each wavelength during each experiment. Data were acquired and analyzed by Felix software (Version 1.4, Photon Technology International). Ca^{2+} transient amplitudes were the difference between systolic and diastolic Ca^{2+}.

2.3. Western Blotting. The expressions of cTnI, total and Ser-16-phosphorylated and Thr-17-phosphorylated PLB were
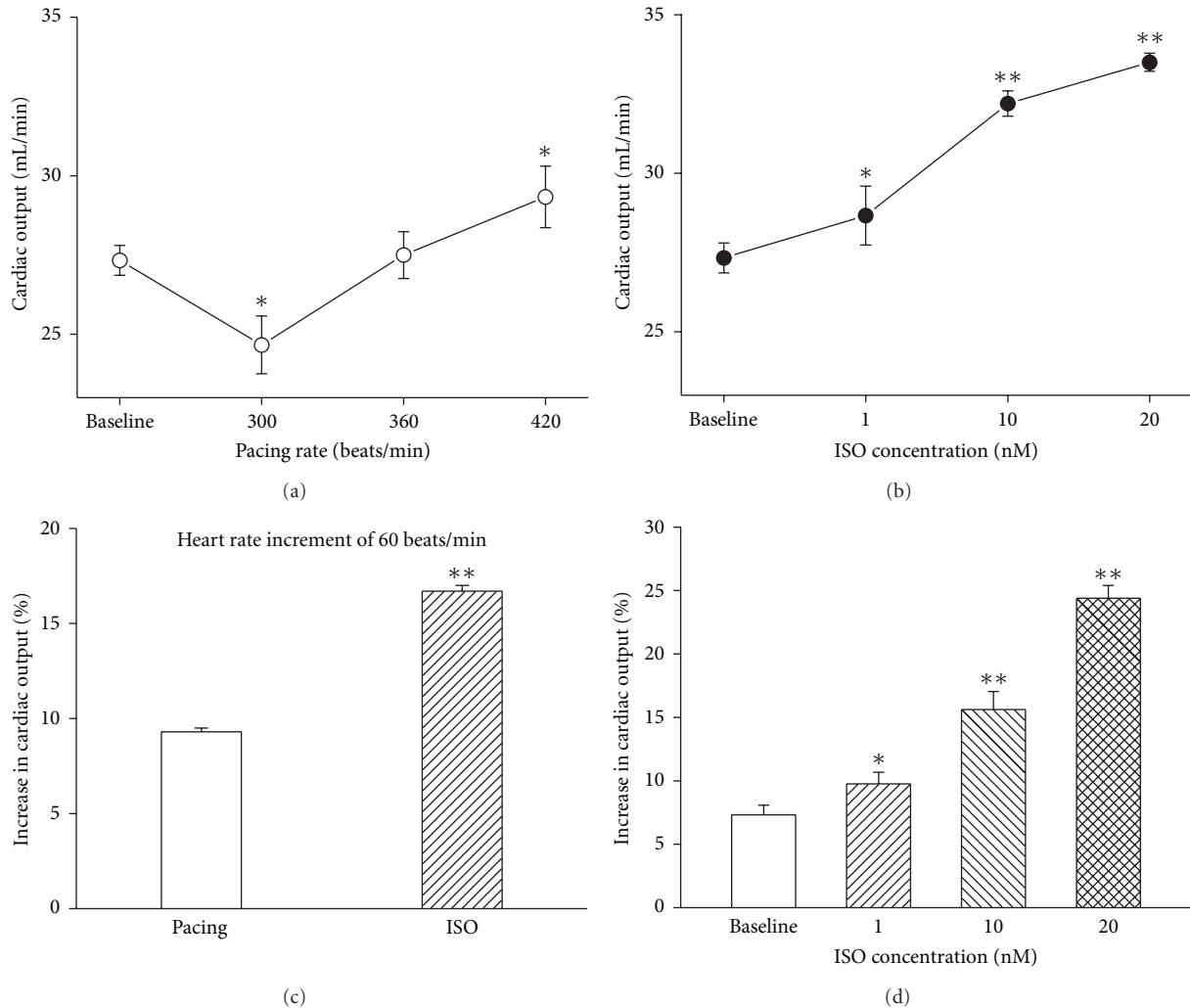
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FIGURE 1: Cardiac output of isolated working hearts in the pacing and ISO treatment. All data are mean ± SEM. (a) Cardiac output of isolated working hearts in different rates of pacing. The $n = 6$ hearts. $^*P < 0.05$ versus baseline. (b) Cardiac output in different concentrations of ISO treatment. The $n = 6$ hearts in each ISO concentration. $^*P < 0.05$ or $^{**}P < 0.01$ versus baseline. (c) The percentage increases in cardiac output in the heart rate increment of 60 beats/min. The $n = 6$ hearts for each group. $^*P < 0.05$ versus the pacing group. (d) The percentage increases in cardiac output at the pacing of 420 beats/min without and with ISO treatment. The $n = 6$ hearts in each ISO concentration. $^*P < 0.05$ or $^{**}P < 0.01$ versus baseline.

detected by Western blotting. Left ventricular myocardium was homogenized in a buffer containing 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM DTT, 1 mM EDTA, 0.3 mM PMSF, and phosphatase inhibitor cocktail (1 : 100, Sigma-Aldrich). Samples were subjected to SDS-PAGE in polyacrylamide gels (12% or 14% depending on protein molecular weight). After electrophoresis, protein was electrically transferred to nitrocellulose membrane (0.45 μm pore size) using a Bio-Rad semidry transfer apparatus. The blotted nitrocellulose membranes were blocked with 1% bovine serum albumin in Tris-buffered saline (150 mM NaCl, 50 mM Tris–HCl, and pH 7.5) and incubated with mouse monoclonal anti-TnI (TnI-1, 1 : 4,000; Courtesy of Dr. J.-P. Jin), mouse monoclonal anti-PLB (1 : 1000; Cell Signaling Technology, Inc. (CST), Danvers, Mass, USA), rabbit polyclonal anti-Ser-16-phosphorylated phospholamban (Phospho16-PLB, 1 : 1000; CST) and rabbit polyclonal anti-Thr-17-phosphorylated phospholamban (Phospho17-PLB, 1 : 1000; Santa Cruz Biotechnology, Inc., Santa Cruz, Calif, USA) in TBS containing 0.1% BSA at 4°C overnight. The membranes were visualized with IRDye 680CW goat antimouse or IRDye 800CW goat antirabbit secondary antibodies (1 : 10,000, for 90 min at room temperature) using an Odyssey scanner (LI-COR Biosciences, Lincoln, Neb, USA). Quantification analysis of blots was performed with the Scion Image software.

2.4. Statistical Analysis. Data are expressed as mean ± SEM. Differences between groups were evaluated using one-way analysis of variance (ANOVA). When ANOVA indicated significance, a Dunnett post-hoc test was performed. $P < 0.05$ was considered statistically significant.
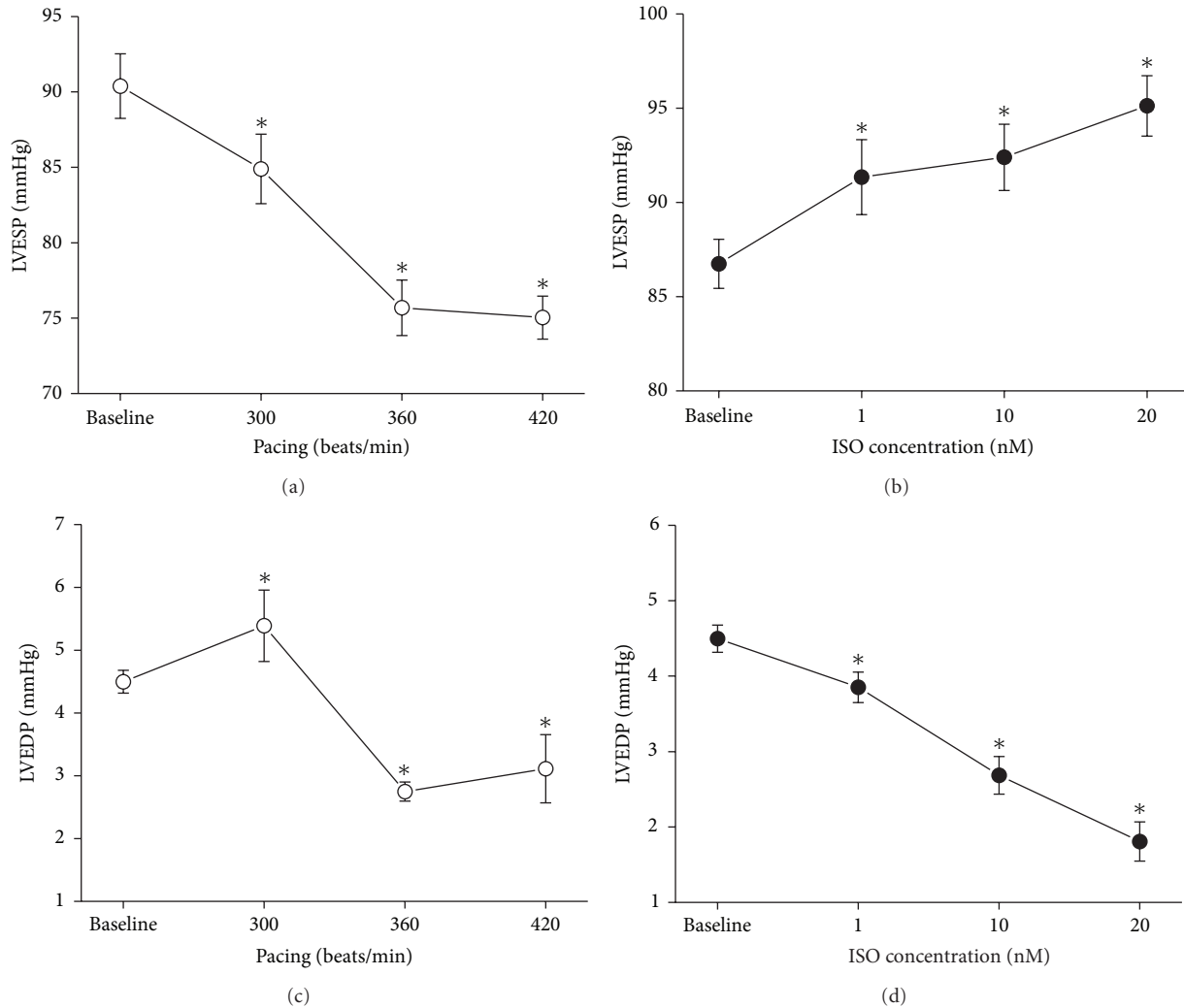
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FIGURE 2: Left ventricular end-systolic pressure (LVESP) and end-diastolic pressure (LVEDP) of working hearts in the pacing and ISO treatment. (a) and (c) LVESP and LVEDP during the pacing. (b) and (d) LVESP and LVEDP in ISO treatment. Data are mean ± SEM, $n = 6$ hearts for each ISO concentration in each group. $^*P < 0.05$ versus baseline.

3. Results

3.1. Systolic and Diastolic Function of Isolated Working Hearts during the Pacing and ISO Treatment. At the preload of 10 mmHg and afterload of 60 mmHg, the intrinsic heart rate of working heart was 236.4 ± 6.4 beats/min in the baseline group. Heart rates increased to 259.9 ± 7.3, 288.1 ± 4.1, and 316.7 ± 6.3 beat/min in 1, 10, and 20 nM ISO stimulation, respectively. At baseline, cardiac output was 27.3 ± 0.5 mL/min. Cardiac output decreased to 24.7 ± 0.9 mL/min at the pacing of 300 beats/min, then increased to 27.5 ± 0.7 mL/min and 29.3 ± 0.9 mL/min at the pacing of 360 and 420 beats/min (Figure 1(a)). Cardiac output increased to 28.7 ± 0.9, 32.2 ± 0.4, and 33.5 ± 0.3 mL/min in 1, 10, and 20 nM ISO treatment, respectively, (Figure 1(b)). While the increment of heart rate was similar at 60 beats/min, the percentage increase in cardiac output was higher in ISO-treated group than in the pacing group (Figure 1(c)). Cardiac output had about 7%, 10%, 15%, and 24% increases,

respectively, at the pacing of 420 beats/min without and with 1, 10, and 20 nM ISO treatment compared to the baseline at the intrinsic heart rate (Figure 1(d)).

LVESP decreased with the increasing pacing rate (Figure 2(a)). In the opposite, LVESP increased with the increasing concentrations of ISO at the intrinsic heart rate (Figure 2(b)). LVEDP showed a significant increase at 300 beats/min but decreased progressively at 360 and 420 beats/min (Figure 2(c)). LVEDP decreased progressively with the increasing concentrations of ISO at the intrinsic heart rate (Figure 2(d)).

The $\pm dP/dt_{max}$ decreased significantly at the pacing of 300 beats/min and increased at the pacing of 360 and 420 beats/min (Figure 3(a)). In contrast, $\pm dP/dt_{max}$ elevated progressively with the increasing concentrations of ISO (Figure 3(b)). TR$_{75}$, time from peak to 75% relaxation of left ventricular pressure, is another index of cardiac diastolic function. Both the pacing and ISO stimulation reduced TR$_{75}$ (Figures 3(c) and 3(d)).
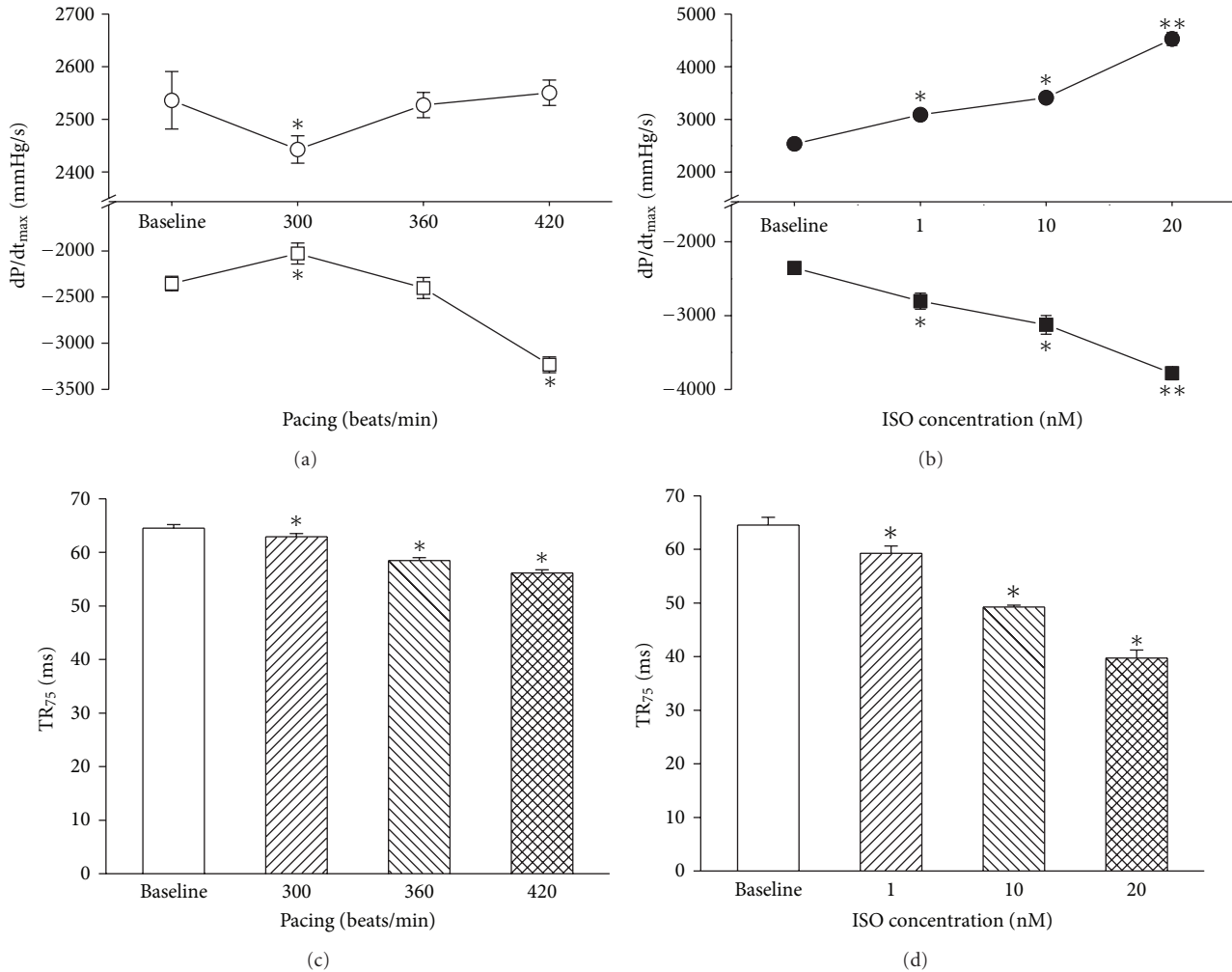
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FIGURE 3: Maximal rates of left ventricular pressure development and relaxation ($\pm dP/dt_{max}$) and time from peak to 75% relaxation (TR_{75}) of left ventricular pressure. (a) and (c) $\pm dP/dt_{max}$ and TR_{75} under different rates of pacing. (b) and (d) $\pm dP/dt_{max}$ and TR_{75} in different concentrations of ISO treatment. Data are mean $\pm$ SEM, $n = 6$ hearts for each ISO concentration in each group. $^*P < 0.05$ or $^{**}P < 0.01$ versus baseline.

The percentage increases in $\pm dP/dt_{max}$ between the pacing of 420 beats/min and intrinsic heart rate groups without ISO stimulation were $7.1 \pm 1.0\%$ and $9.7 \pm 1.1\%$, respectively, (the baseline values in Figure 4(a)). Under ISO stimulation, the pacing of 420 beats/min induced an additional enhancement. The percentage increases in $\pm dP/dt_{max}$ between the pacing of 420 beats/min and intrinsic heart rate groups increased progressively with the increasing concentrations of ISO (Figure 4(a)). The pacing of 420 beats/min led about 13% shortening in TR_{75} without ISO stimulation. Under ISO treatment, the pacing of 420 beats/min showed stronger relaxant effects than that of the baseline group (Figure 4(b)).

3.2. Intracellular Ca^{2+} Concentrations of the Cardiomyocyte in the Pacing and ISO Treatment.

The resting intracellular calcium concentration ($[Ca^{2+}]_i$) increased with the increasing frequency of stimulation; higher frequency of stimulation (i.e., >5 Hz) induced a significant elevation in resting $[Ca^{2+}]_i$

of cardiomyocytes (Figure 5(a)). The peak $[Ca^{2+}]_i$ increased to a maximal level at 5 Hz, then decreased slightly at 10 Hz. In contrast, the resting $[Ca^{2+}]_i$ decreased slightly, but the peak $[Ca^{2+}]_i$ kept elevating with the increasing concentrations of ISO (Figure 5(b)). Under ISO perfusion, the increasing degrees of resting $[Ca^{2+}]_i$ were less compared with non ISO-treated group while the frequency of stimulation increased from 300 to 420 beats/min (Figure 5(a)). The peak $[Ca^{2+}]_i$ still elevated with the increasing frequency of stimulation during different concentrations of ISO treatment. The peak $[Ca^{2+}]_i$ reached the maximal level at 10 Hz under 20 nM ISO treatment (Figure 5(a)).

TD_{75}, the time from peak to 75% decay in calcium transient, reduced both under increasing frequency of stimulation and ISO perfusion. The degrees of reduction in TD_{75} were lower in stimulation group than in ISO-treated group (Figures 5(c) and 5(d)).

3.3. The Phosphorylation of cTnI at Ser23/24 and PLB at Ser16 and Thr17 in the Pacing and ISO Treatment.
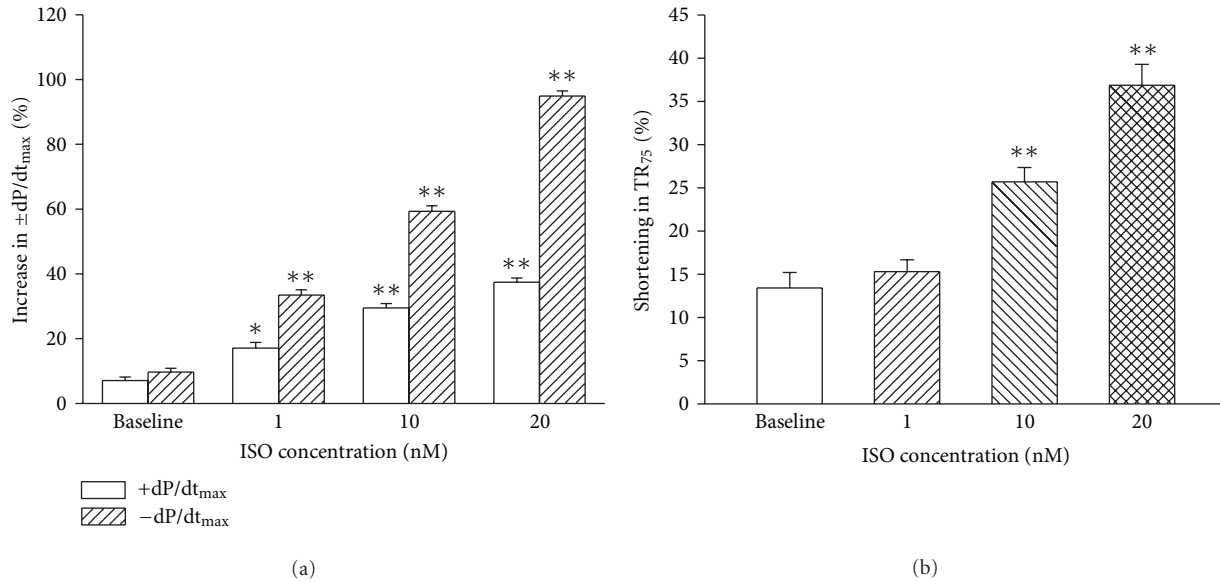
Figure 6(a)

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(b)

FIGURE 4: Percentage increases in $\pm dP/dt_{max}$ and TR_{75} under combined effect of the pacing and ISO. (a) The percentage increases in $\pm dP/dt_{max}$ between the pacing of 420 beats/min and intrinsic heart rate without and with ISO treatment. (b) The percentage shortenings in TR_{75} between the pacing of 420 beats/min and intrinsic heart rate without and with ISO treatment. Data are mean $\pm$ SEM, $n = 6$ hearts for each ISO concentration in each group. $*P < 0.05$ or $**P < 0.01$ versus baseline.

shows the representative Western blots of cTnI and phosphorylated cTnI at Ser23/24. There were no differences in expression of cTnI in the pacing, ISO treatment, and pacing plus ISO treatment groups (Figure 6(b)). Phosphorylation of cTnI at Ser23/24 showed a dose-dependent increase in ISO perfusion. But the pacing had no effect on phosphorylation of cTnI at Ser23/24 in basic and ISO-treated condition (Figure 6(c)).

Figure 7(a) shows the representative Western blots of PLB, phosphorylated PLB at Ser16, and Thr17 in isolated working hearts under the pacing and different concentrations of ISO perfusion. Total PLB, which is the sum of pentameric and monomeric PLB, did not change under the pacing and ISO perfusion (Figure 7(b)). The phosphorylation of PLB at Ser16 and Thr17 also showed a dose-dependent increase in ISO stimulation. The degrees of phosphorylated PLB at Ser16 elevated from $39.3 \pm 1.4\%$ in baseline to $50.2 \pm 1.7\%$ in 20 nM ISO treatment, and the degrees of phosphorylated PLB at Thr17 increased from $13.9 \pm 0.6\%$ in baseline to $22.5 \pm 0.6\%$ in 20 nM ISO treatments (Figures 7(c) and 7(d)). The phosphorylation of PLB at Thr17 increased with the increasing pacing rate, but the pacing did not alter the phosphorylation of PLB at Ser16. During 10 nM and 20 nM ISO perfusion, the pacing induced an additional increase in the phosphorylation of PLB at Ser16 in a frequency-dependent manner (Figure 7(d)).

4. Discussion

4.1. Coordinated Activation of Ca^{2+} Handing Proteins in Excitation-Contraction Coupling Is Important to Considerably Accelerate Relaxation in ISO Stimulation. In the present study, the increase in maximal rate of relaxation and the

shortening in TR_{75} were lower in the pacing than in ISO stimulation (Figure 4(a)). It indicates that the extent of FDAR is less than that of IDAR. FDAR is an important intrinsic regulator to cardiac function in physiological condition. However, the mechanism of FDAR is still controversial. Some studies reported that FDAR may be related to the activation of CaMKII, phosphorylation of PLB, and the increase of SERCA activity. There are evidences to indicate that inhibition of CaMKII by KN-93 [9], CaMKII and PLB knock-out [11, 19], or inhibition of SERCA also shows an acceleration of relaxation in the increasing stimulation frequency [11]. We observed that the pacing only increased the phosphorylation of PLB at Thr-17 site (Figure 7). The increase in Thr-17 phosphorylation of PLB was associated with FDAR, but the relaxation effect was weak. The Thr-17 phosphorylation of PLB can relieve the inhibition to SERCA. Since the activation of SERCA by Thr-17 phosphorylation of PLB is low, the resting intracellular Ca^{2+} concentration slightly elevated with an increasing stimulation frequency (Figure 5(a)). In contrast, ISO treatment showed not only the phosphorylation of PLB at Ser-16 and Thr-17 sites, but also the cTnI phosphorylation (Figure 7(a)). Several researches have indicated that PLB phosphorylation by ISO stimulation is a dominant mediator to the acceleration of relaxation in PLB knock-out mouse [20]. But it should not be ignored that the effect of cTnI phosphorylation was induced by ISO treatment on the acceleration of relaxation.

Early investigations indicate that phosphorylation of cTnI may not be important in the relaxant effect of ISO stimulation. Johns et al. found no effect of cTnI phosphorylation by PKA on the relaxation in skinned guinea pig ventricular trabeculae [21], and the precise experimental conditions may be important in this regard. Recently, many studies
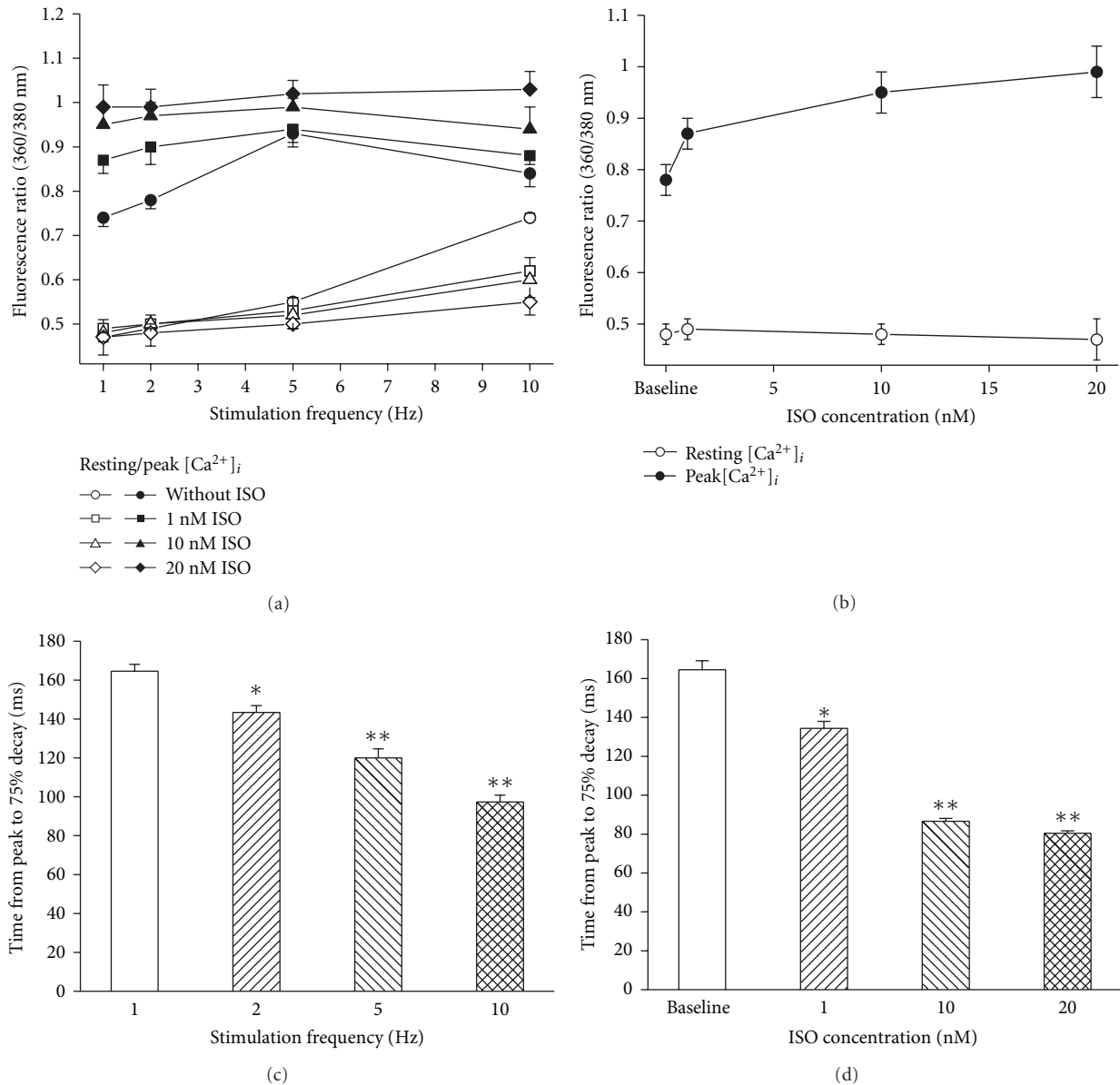
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FIGURE 5: Resting and peak calcium concentrations in cardiomyocytes and time from peak to 75% decay (TD_{75}) under different frequencies of stimulations with and without ISO treatment. Data are mean ± SEM, n = at least 30 myocytes from six hearts for each concentration in each group. At least 5 myocytes were from each heart. $*P < 0.05$ or $**P < 0.01$ versus baseline.

indicate that phosphorylation of cTnI by PKA may be particularly important in modulating diastolic function of hearts. Fentzke et al. constructed transgenic mice (TG-ssTnI); the myocardium expressed slow skeletal TnI (ssTnI) which lacks N-terminal extension containing two PKA phosphorylation sites. They reported that in situ relaxation of left ventricular pressure was significantly slowed in TG-ssTnI hearts compared with the wild-type mice [22]. Kentish et al. also studied transgenic mice with ssTnI replacing cTnI and used flash photolysis of diazo-2 to rapidly remove Ca^{2+} from skinned cardiac muscle fibers. Myocardium expressing ssTnI had no acceleration of relaxation in β-adrenergic stimulation, suggesting that cTnI phosphorylation played an important role in intrinsic relaxation [23]. Li et al. reported the first

quantitative estimates of the relative contributions of PLB and TnI phosphorylation to the ISO induced acceleration of relaxation in mouse hearts. The TnI phosphorylation may contribute up to 14–18% of the acceleration of relaxation in response to ISO during isometric contractions in mouse myocardium [20]. Wolska et al. observed four lines of mice expressed PLB/cTnI, PLB/ssTnI, PLBKO (PLB knockout)/cTnI, and PLBKO/ssTnI in the heart, respectively. They demonstrated that expression of ssTnI in hearts of PLB knock-out mice altered the relaxant effect of β-adrenergic stimulation [24]. The underlying mechanisms of phosphorylation of cTnI by PKA regulating relaxation of hearts may be involved in myofibril Ca^{2+} sensitivity [16], an increase in crossbridge cycling [23], and increased binding of cTnI
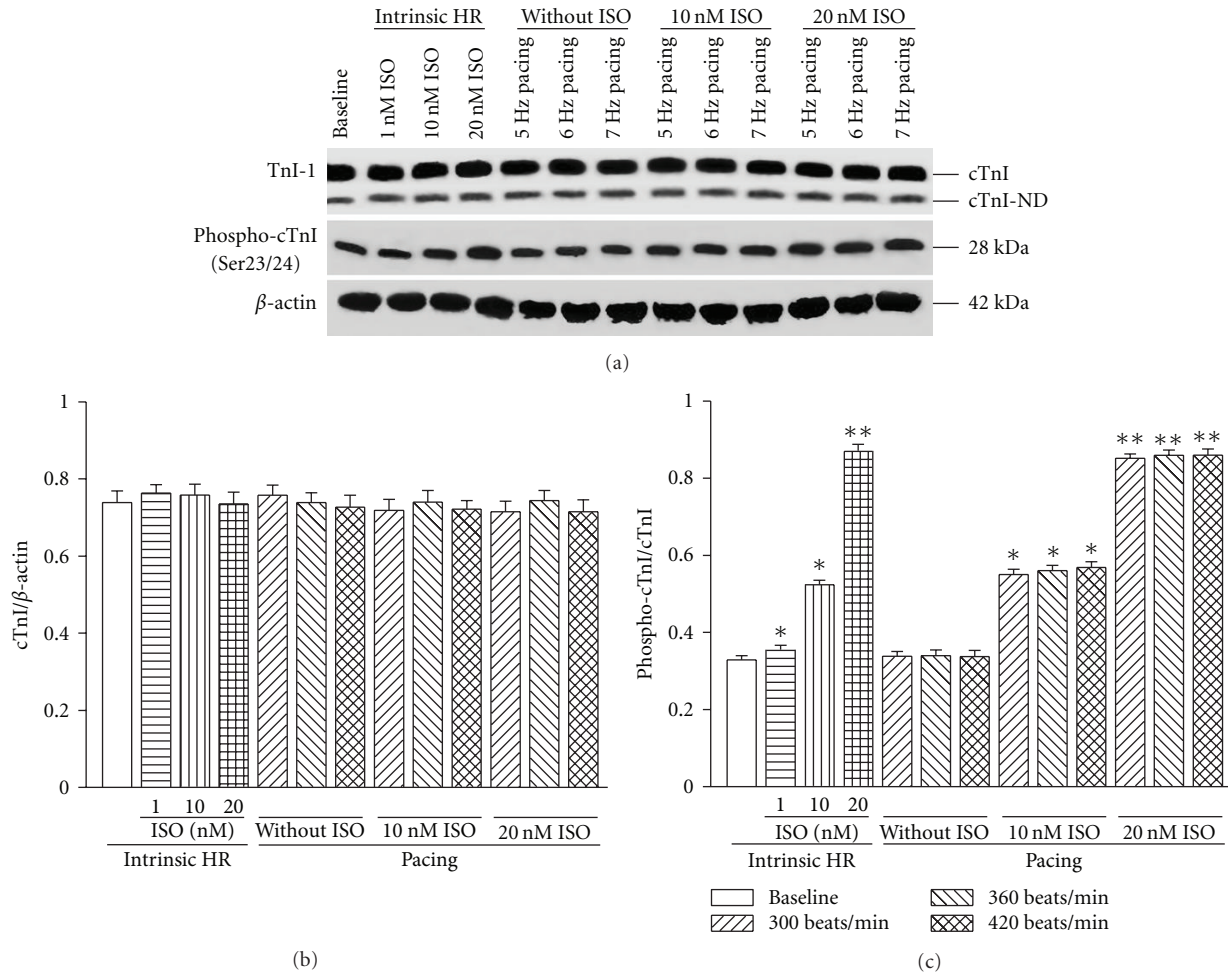
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FIGURE 6: Expressions of cTnI and phosphorylated cTnI at Ser23/24 in the myocardium after the pacing and ISO treatment. (a) The representative Western blots of cTnI and phosphorylated cTnI at Ser23/24. (b) Expression of total cTnI which is the sum of cTnI and NH_2-terminal deleted fragment of cTnI (cTnI-ND). (c) The phosphorylation of cTnI. Data are mean ± SEM, $n = 6$ hearts in each group. $*P < 0.05$ or $**P < 0.01$ versus baseline.

to the thin filament. The phosphorylation of cTnI reduces Ca^{2+} sensitivity of myofibrils which accelerates the rate of Ca^{2+} dissociation from cardiac troponin C. Taken together, phosphorylation of PLB and cTnI induces a synergistic effect to accelerate relaxation in ISO administration. Therefore, IDAR is stronger than FDAR. The less extent of FDAR elevates LVEDP which induces an insufficient diastolic filling at higher heart rate and further may reduce cardiac output.

4.2. The Phosphorylation of cTnI Causes a Synergistic Effect between PLB and cTnI and Further Enhances the Relaxation in Increasing Frequency of Stimulation. Although PKA phosphorylates many sites in the cardiomyocyte, only phosphorylated PLB and cTnI are directly related to the acceleration of relaxation in ISO stimulation. Due to the synergistic effect of PLB and cTnI, the degree of relaxation in IDAR is higher than that in FDAR. In this study, the degrees of relaxation in increasing rate of pacing showed the additional increases during ISO perfusion. Varian and Janssen reported that FDAR involved decreased myofilament calcium sensitivity

[25]. Phosphorylation of cTnI can reduce myofilament calcium sensitivity. It suggests that phosphorylation of cTnI would be helpful to FDAR. Takimoto et al. generated transgenic mice that overexpress cTnI in which the serine residues normally targeted by PKA were mutated to aspartic acid to mimic constitutive phosphorylation (cTnI-$P_{22}P_{23}$). The cTnI-$P_{22}P_{23}$ hearts showed a frequency-dependent enhancement of systolic and diastolic in vivo and afterload sensitivity of relaxation [26]. Using similar transgenic mice, Sakthivel et al. also demonstrated that the cTnI-$P_{22}P_{23}$ hearts showed augmented relaxation and contraction at higher heart rates compared with the nontransgenic controls [16].

The 10 nM of ISO induced about 50% phosphorylation of cTnI, 45% phosphorylation of PLB at Ser-16 site, and 14% phosphorylation of PLB at Thr-17 site in the present study. These results are in agreement with the experiments of Kuschel and colleagues [27]. The phosphorylation of cTnI by ISO stimulation showed an enhancement in the extent of FDAR compared with that in the absence of ISO. While 90% of the cTnI was phosphorylated in
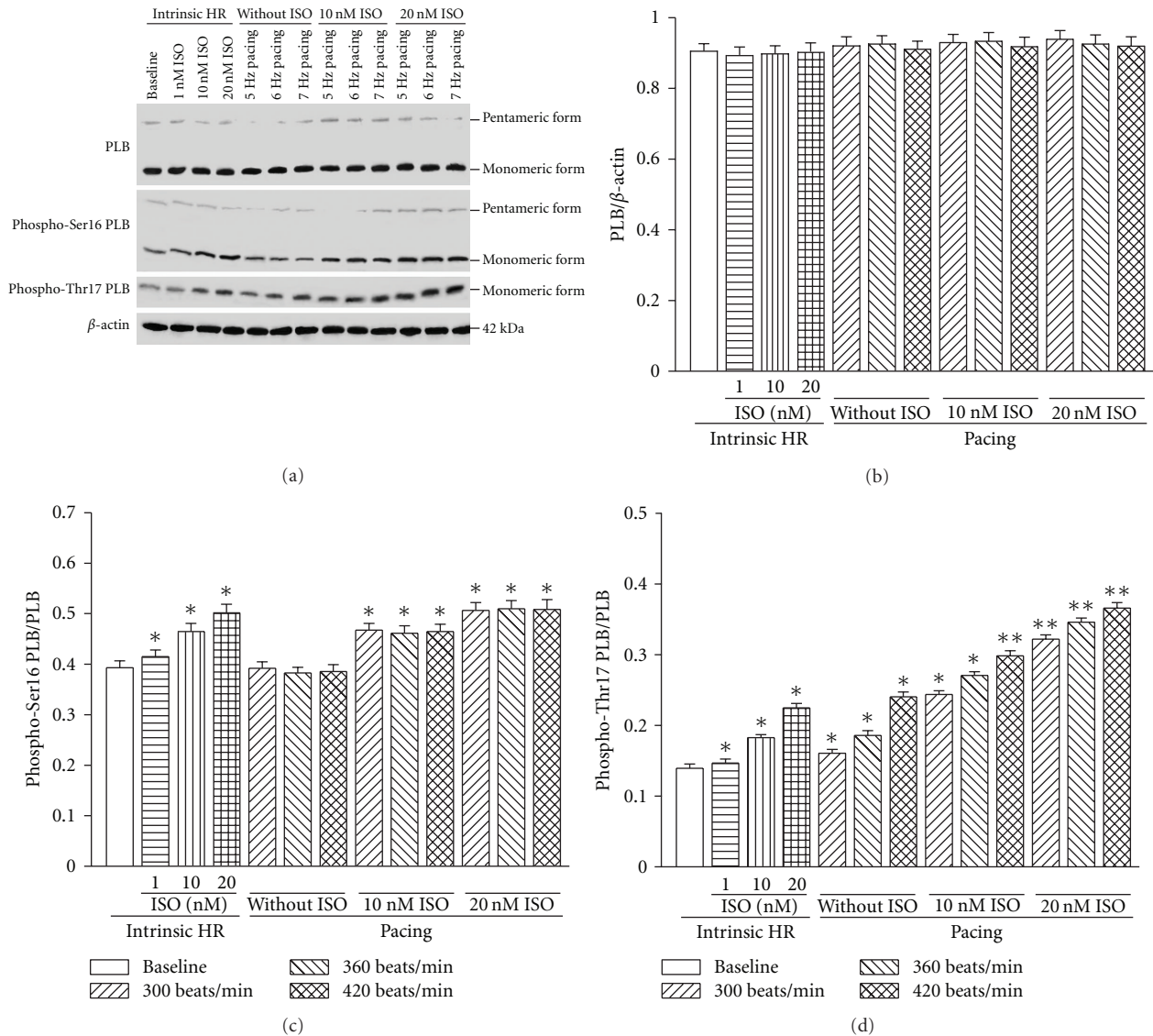
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FIGURE 7: Expression of PLB, phosphorylated PLB at Ser16 (phospho Ser16-PLB) and at Thr17 (phospho Thr17-PLB) in the myocardium after the pacing and ISO treatment. (a) The representative Western blots of PLB, phosphorylated PLB at Ser16 and Thr17. (b) Expression of total PLB measured by pentametric plus monomeric forms. (c) Expression of phospho Ser16-PLB. (d), expression of phospho Thr17-PLB. Data are mean ± SEM, $n = 6$ hearts in each group. $*P < 0.05$ or $**P < 0.01$ versus baseline.

20 nM of ISO treatment, the PLB Ser-16 and Thr-17 had 50% and 22% phosphorylation, respectively. The extent of FDAR in the presence of 20 nM ISO further increased. The additional enhancement in phosphorylation of PLB Thr-17 may form a synergistic effect with phosphorylation of cTnI by PKA with increasing stimulation frequency, and hence, the extent of FDAR increased. Finally, cardiac output increased in the presence of ISO rather than decreased in the absence of ISO.

In summary, this study provides evidence supporting an important role for cardiac TnI phosphorylation by PKA in the enhancement of diastolic function. And the synergistic effects between the PLB and cTnI are necessary in promoting relaxation, especially at higher heart rate.

Conflicts of Interests

The authors declare that there is no conflict of interests.

Acknowledgments

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