Both Ser$^{16}$ and Thr$^{17}$ of phospholamban (PLB) are phosphorylated, respectively, by cAMP-dependent protein kinase (PKA) and Ca$^{2+}$/calmodulin-dependent protein kinase II (CaMKII). PLB phosphorylation relieves cardiac sarcoplasmic reticulum Ca$^{2+}$ pump from inhibition by PLB. Previous studies have suggested that phosphorylation of Ser$^{16}$ by PKA is a prerequisite for Thr$^{17}$ phosphorylation by CaMKII and is essential to the relaxant effect of β-adrenergic stimulation. To determine the role of Thr$^{17}$ PLB phosphorylation, we investigated the dual-site phosphorylation of PLB in isolated adult rat cardiac myocytes in response to β$_{1}$-adrenergic stimulation or electrical field stimulation (0.1–3 Hz) or both. A β$_{1}$-adrenergic agonist, norepinephrine (10$^{-5}$–10$^{-6}$ M), in the presence of an α$_{1}$-adrenergic antagonist, prazosin (10$^{-6}$ M), selectively increases the PKA-dependent phosphorylation of PLB at Ser$^{16}$ in quiescent myocytes. In contrast, electrical pacing induces an opposite phosphorylation pattern, selectively enhancing the CaMKII-mediated Thr$^{17}$ PLB phosphorylation in a frequency-dependent manner. When combined, electric stimulation (2 Hz) and β$_{1}$-adrenergic stimulation lead to dual phosphorylation of PLB and exert a synergistic effect on phosphorylation of Thr$^{17}$ but not Ser$^{16}$. Frequency-dependent Thr$^{17}$ phosphorylation is closely correlated with a decrease in 50% relaxation time ($t_{50}$) of cell contraction, which is independent of, but additive to, the relaxant effect of Ser$^{16}$ phosphorylation, resulting in hastened contractile relaxation at high stimulation frequencies. Thus, we conclude that in intact cardiac myocytes, phosphorylation of PLB at Thr$^{17}$ occurs in the absence of prior Ser$^{16}$ phosphorylation, and that frequency-dependent Thr$^{17}$ PLB phosphorylation may provide an intrinsic mechanism for cardiac myocytes to adapt to a sudden change of heart rate.

Phospholamban (PLB)$^{1}$ is the major regulator of cardiac sarcoplasmic reticulum (SR) Ca$^{2+}$-ATPase and Ca$^{2+}$ transport across the SR membrane, thereby modulating myocardial relaxation (for review see Ref. 1). Specifically, PLB in its dephosphorylated state is an inhibitor of the SR Ca$^{2+}$ pump (2). Phosphorylation of PLB at Ser$^{16}$ and Thr$^{17}$ by PKA and CaMKII removes this inhibition by increasing the affinity of the pump for Ca$^{2+}$ (1–3). In the beating mammalian heart, β-adrenergic stimulation increases both PKA- and CaMKII-mediated phosphorylation of Ser$^{16}$ and Thr$^{17}$ (4–6). More recent studies have shown that the effects of β-adrenergic stimulation on PLB phosphorylation are mostly attributable to β$_{1}$- but not β$_{2}$-adrenergic receptor subtype (7–10).

Over the last two decades, intensive studies have been focused on the physiological significance of the dual site phosphorylation of PLB. These previous studies in perfused hearts or in vivo have provided several lines of evidence leading to the concept that Ser$^{16}$ phosphorylation is a prerequisite for phosphorylation of Thr$^{17}$ and that Ser$^{16}$ phosphorylation is largely responsible for β-adrenergic modulation of cardiac relaxation (4, 5, 11, 12, 14–18). Recent studies in transgenic mice overexpressing the Ser$^{16}$ → Ala$^{16}$ PLB mutant have further demonstrated that prevention of Ser$^{16}$ phosphorylation abolishes Thr$^{17}$ phosphorylation and attenuates β-adrenergic responses (19). Thus, it is widely accepted that phosphorylation of PLB at Ser$^{16}$ is obligatory for Thr$^{17}$ phosphorylation and that Ser$^{16}$ phosphorylation is the dominant molecular event responsible for accelerated cardiac relaxation. However, in vitro studies in the isolated SR membranes have consistently indicated that Ser$^{16}$ and Thr$^{17}$ can be readily and independently phosphorylated by PKA and CaMKII, respectively, and that when both are phosphorylated, there is an additive interaction (20, 21). The apparent discrepancy between in vivo and in vitro PLB phosphorylation is yet to be reconciled.

To resolve this paradox and to further address the relative contribution of PKA- and CaMKII-mediated PLB phosphorylation in heat-to-beat cardiac functional modulation, individually we manipulated PKA activity, using β-adrenergic stimulation in quiescent rat ventricular myocytes, and CaMKII activity, by electrically pacing the myocytes at different stimulation frequencies (0.1–3 Hz) in the absence of β-adrenergic stimulation. Both stimuli were also combined to explore possible interactions between PKA- and CaMKII-mediated signaling. Under those experimental conditions, we measured PLB phosphorylation at Ser$^{16}$ and Thr$^{17}$ as well as relaxation time of cell contraction. Here, we report our surprising findings that electrical stimulation alone increases CaMKII-dependent phosphorylation of PLB at Thr$^{17}$ in a frequency-dependent manner without altering PKA-mediated Ser$^{16}$ phosphorylation, that phosphorylation of Thr$^{17}$ is markedly enhanced by β-adrenergic stimulation in the electrically paced but not in quiescent myocytes, and that Thr$^{17}$ phosphorylation is associated with a significant relaxant effect, regardless of β-adrenergic stimulation.

---

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Laboratory of Cardiovascular Science, Gerontology Research Center, NIA, National Institutes of Health, 5600 Nathan Shock Dr., Baltimore, MD 21224. Tel.: 410-558-8662; Fax: 410-558-8150; E-mail: XiaoR@grc.nia.nih.gov.

‡ The abbreviations used are: PLB, phospholamban; PKA, cAMP-dependent protein kinase; SR, sarcoplasmic reticulum; NE, norepinephrine; PP1, protein phosphatase 1; CaMKII, calmodulin-dependent protein kinase II.

Received for publication, April 12, 2000, and in revised form, May 19, 2000 Published, JBC Papers in Press, May 23, 2000, DOI 10.1074/jbc.C000253200
EXPERIMENTAL PROCEDURES

Measurements of Cell Contraction and Relaxation—Single ventricular myocytes were isolated from adult rat (2-4-month old) hearts by a standard enzymatic technique (22). The cells were suspended in HEPES buffer, pH 7.4, containing (in mmol/liter): 20 HEPES, 1 CaCl₂, 137 NaCl, 5 KCl, 15 dextrose, 1.3 MgSO₄, and 1.2 NaH₂PO₄. Cells were kept at rest or stimulated at different frequencies ranging from 0.1 to 3 Hz at 23°C, and cell length was monitored from the bright-field image by an optical edge tracking method using a photodiode array (model 1024 SAQ, Reticon) with a 3-ms time resolution (22). The half-time of contractile relaxation (t₅₀) was measured as the time from cell peak shortening to 50% relaxation.

Site-specific PLB Phosphorylation—The detection of site-specific PLB phosphorylation was performed as described previously (9). Briefly, a 500-μl suspension of isolated rat ventricular myocytes was stimulated over a wide range of frequencies (0.1–3 Hz) at 23°C. Following a 5-min stimulation, 4× sample buffer was added, and the samples were frozen in liquid nitrogen. For β₁-adrenergic stimulation, myocytes were incubated with norepinephrine (NE, 10⁻⁶–10⁻⁸ M) and prazosin (10⁻⁶ M) for 10 min. In another subset of experiments, myocytes were first incubated with NE (10⁻⁷ M) plus prazosin (10⁻⁶ M) for 5 min and then were stimulated electrically (2 Hz) for another 5 min in the continued presence of β₁-adrenergic stimulation. Samples were solubilized prior to electrophoresis at 95°C for 5 min to fully dissociate PLB into its monomers. Following electrophoresis, proteins were transferred to a polyvinylidene difluoride membrane (PVDF, Sigma), which was probed with the phosphorylation site-specific PSer₁⁶ and PThr₁⁷ PLB antibodies (PhosphoProtein Research). Protein concentration was determined by the method of Lowry et al. (23) using ovalbumin as standard. Following incubation with a peroxidase-conjugated antibody (Dianova), films were exposed to the chemiluminescence (ECL, Amersham Pharmacia Biotech) reaction and quantified with a video documentation system (Bio-Rad).

Statistics and Other Assays—Results are presented as the mean ± S.E. Statistical significance was determined by Student’s t test, the Mann-Whitney test (when variances were significantly different), or one-way analysis of variance, when appropriate. Values with p < 0.05 were considered statistically significant.

RESULTS

Dose-Response of NE-induced Ser₁⁶ PLB Phosphorylation—Freshly isolated single rat quiescent ventricular myocytes were challenged by NE (10⁻⁶–10⁻⁸ M), a β₁-adrenergic receptor agonist, in the presence of an α₁-adrenergic antagonist, prazosin (10⁻⁶ M). Phosphorylation of Ser₁⁶ or Thr₁⁷ in response to β₁-adrenergic stimulation was determined by immunoblots with anti-PSer₁⁶ PLB and anti-PThr₁⁷ PLB antibodies. As shown in Fig. 1, NE increased PKA-mediated PLB phosphorylation at Ser₁⁶ in a dose-dependent manner, with an EC₅₀ of ~10⁻⁸ M. In contrast, NE, even at the maximal concentration (10⁻⁶ M), had only a very minor effect on CaMKII-dependent phosphorylation of Thr₁⁷ (Fig. 1).

Frequency-dependent Thr₁⁷ PLB Phosphorylation in the Absence of β-Adrenergic Stimulation—To examine PLB phosphorylation at the CaMKII site Thr₁⁷, myocytes were electrically paced for 5 min at frequencies ranging from 0.1 to 3.0 Hz. Fig. 2 shows the response of Ser₁⁶ or Thr₁⁷ PLB phosphorylation to stimulation frequency in the absence of β-adrenergic stimulation. There was a near linear increase in Thr₁⁷ PLB phosphorylation with increasing stimulation frequency, and the phosphorylation was not yet saturated at the highest stimulation frequency (147.70 ± 13.98 at 3 Hz versus 44.07 ± 11.55 at rest, p < 0.01). In the same cells, however, no significant PKA-mediated PLB phosphorylation at Ser₁⁶ was observed at any stimulation frequency. The pattern of Ser₁⁶ and Thr₁⁷ phosphorylation induced by electrical stimulation alone was thus opposite to that in quiescent cells induced by β-adrenergic stimulation, suggesting that CaMKII-mediated Thr₁⁷ PLB phosphorylation can occur independent of any prior Ser₁⁶ PLB phosphorylation in intact cardiac myocytes.

Ser₁⁶ and Thr₁⁷ PLB Phosphorylation in Response to Combined Electrical and β-Adrenergic Stimulation—To further investigate possible interactions between the PKA and CaMKII phosphorylation sites, we measured site-specific PLB phosphorylation using electrical stimulation in conjunction with β₁-adrenergic stimulation. Following a 5-min incubation with 10⁻⁶ M NE plus 10⁻⁶ M prazosin, myocytes were either kept at rest or electrically paced at 2 Hz for another 5 min in the continued presence of NE. Electrical stimulation had no significant effect on NE-induced Ser₁⁶ PLB phosphorylation (Fig. 3A). In contrast, β₁-adrenergic stimulation markedly augmented the frequency-dependent PLB phosphorylation at Thr₁⁷, resulting in a 3-fold augmentation compared with that in the absence of β₁-adrenergic stimulation (Fig. 3B). Thus, there is a synergistic interaction between electrical stimulation and β₁-adrenergic stimulation in conferring Thr₁⁷ PLB phosphorylation, whereas the effect of β₁-adrenergic stimulation on Ser₁⁶ phosphorylation is independent of electrical stimulation.

Modulatory Effect of Stimulation Frequency on Cardiac Relaxation—To determine the functional role of Thr₁⁷ PLB phosphorylation, we examined the t₅₀ of contraction in the absence as well as in the presence of β₁-adrenergic stimulation in rat cardiomyocytes. We found that in the absence of β₁-adrenergic stimulation, t₅₀ after a 5-min pacing period was abbreviated relative to t₅₀ of the first post-rest beat. The higher the pacing frequency, the briefer the t₅₀ of relaxation (t₅₀ values: 326.5 ± 11.32 ms for post-rest; and 299.74 ± 13.84, 281.16 ± 11.84, 251.19 ± 8.31, 222.23 ± 6.21, and 205.1 ± 5.42 ms for steady state stimulation at 0.1, 0.5, 1.0, 2.0, 3.0 Hz, respectively). Fig. 4C shows that the frequency-dependent abbreviation of 50% relaxation time was linearly correlated to the frequency-dependent increase in Thr₁⁷ PLB phosphorylation but was totally dissociated from Ser₁⁶ phosphorylation (which remained un-
changed during electrical pacing) (Fig. 2). In the presence of β₁-adrenergic stimulation, the first post-rest beat relaxed 14.1% faster relative to NE-untreated cells (Fig. 4B), and this was accompanied by an enhanced PLB phosphorylation occurring exclusively at the PKA site (Figs. 1 and 3A). The relaxant effect of NE was almost doubled by the application of 2 Hz electrical stimulation (Fig. 4). Concomitantly, the enhanced relaxant effect was accompanied by a markedly potentiated Thr¹⁷ phosphorylation (Fig. 4A) in the absence of any change in Ser¹⁶ PLB phosphorylation (Fig. 3A), suggesting that the increased Thr¹⁷ PLB phosphorylation has an additive effect to hasten relaxation during combined β₁-adrenergic and electrical stimulation.

**DISCUSSION**

There are four major findings in the present study. First, β-adrenergic receptor stimulation in quiescent ventricular myocytes increases PKA-dependent phosphorylation of PLB at Ser¹⁶ and accelerates the relaxation rate of the first post-rest beat, with little effect on the CaMKII-dependent phosphorylation of Thr¹⁷ (Fig. 1). This observation is consistent with the previous notion that Ser¹⁶ is the direct and dominant site for PKA-mediated phosphorylation underlying β-adrenergic relaxant effect. Second, and more importantly, in the absence of β-adrenergic stimulation, electrical stimulation enhances phosphorylation of PLB at Thr¹⁷ in a frequency-dependent manner without altering the phosphorylation status of Ser¹⁶ (Fig. 2). These results suggest that phosphorylation states of Ser¹⁶ and Thr¹⁷ can be regulated independently in intact cardiac myocytes. Third, a combination of the β₁-adrenergic agonist, NE (10⁻⁷ M), and electrical stimulation (at 2 Hz) induces dual phosphorylation of PLB at Ser¹⁶ and Thr¹⁷, exhibiting a synergistic effect on Thr¹⁷ but not Ser¹⁶ phosphorylation (Fig. 3). Finally, we provided direct evidence that the frequency-dependent Thr¹⁷ PLB phosphorylation is associated with a significant relaxant effect, independent of β-adrenergic-receptor stimulation (Fig. 4).

**Independent Modulation of PLB Phosphorylation at Ser¹⁶ and Thr¹⁷**—The observations described above indicate that CaMKII-dependent Thr¹⁷ phosphorylation can occur independent of PKA-mediated Ser¹⁶ phosphorylation and is likely involved in the modulation of cardiac relaxation. This conclusion is in contrast to the well accepted sequential model of PLB phosphorylation at Ser¹⁶ and Thr¹⁷ (4, 5, 11, 12, 14–19). Several differences between the present and previous studies may account for the different outcomes. First, our experimental setting permits either a selective PKA activation (β₁-adrenergic stimulation in quiescent cardiac myocytes) or a selective CaMKII activation (electrical stimulation in the absence of β-adrenergic stimulation), avoiding possible cross-talk between PKA and CaMKII signaling pathways. In contrast, most previous studies were performed in vivo, or in isolated beating hearts, and could not distinguish the primary effects of PKA from its secondary effects via interaction with the CaMKII signaling pathway (see below). Additionally, using site-specific antibodies for PSer¹⁶ or PThr¹⁷ PLB with an appropriate exposure time for the chemiluminescence reaction (see “Experimental Procedures”), we have greatly improved the sensitivity to detect a low level phosphorylation of PLB in the present study. Our results indicate that the increase in Thr¹⁷ PLB phosphorylation induced by electrical stimulation (2 Hz) is ~30% of that induced by a combined β-adrenergic and electrical stimulation (~15% of the total phosphorylation of PLB).
This relatively low level of PLB phosphorylation could have been overlooked in previous studies, particularly when it was measured by $^{32}$P incorporation into PLB. It is noteworthy that although previous studies have demonstrated the reliability and specificity of the site-specific antibodies for PSer$^{16}$ and PThr$^{17}$ PLB (12, 16, 24), a recent study (25) shows poor reactivities of those antibodies with dual-phosphorylated PLB. If this were the case, we might underestimate the degree of the sequential PLB phosphorylation. However, this is unlikely, because the present results show that in the presence of both electrical and $\beta$-adrenergic stimuli anti-PSer$^{16}$ and anti-PThr$^{17}$ detected a clear increase in the phosphorylation of both sites (Fig. 3 and 4). Finally, it has been proposed that in vivo or in beating hearts, the dependence of Thr$^{17}$ phosphorylation on prior Ser$^{16}$ phosphorylation is due to a counteraction of protein phosphatases on phosphoproteins (16). In principle, a low level of protein phosphatase activity in freshly isolated myocytes relative to that found in vivo might also explain the discrepancy between this and previous reports (4, 12, 14–18). However, this explanation is unlikely because in quiescent cardiac myocytes $\beta$-adrenergic stimulation increases Thr$^{17}$ PLB phosphorylation only in the presence of protein phosphatase inhibitors (either okadaic acid or calyculin A), suggesting that protein phosphatases are rather active in intact isolated cardiac myocytes.

**Synergistic Effect of $\beta$-Adrenergic and Electrical Stimulation on Thr$^{17}$ Phosphorylation**—It is noteworthy that the increase in phosphorylation at the CaMKII site Thr$^{17}$ in response to a combination of NE and 2 Hz electrical stimulation is much greater than the summation of its separate responses to these two stimuli (Fig. 3), suggesting a synergistic interaction between $\beta$-adrenergic and electrical stimuli. The most plausible explanation is that $\beta$-adrenergic stimulation indirectly enhances Thr$^{17}$ phosphorylation through cross-talking with CaMKII signaling pathway. For instance, $\beta$-adrenergic stimulation elevates CaMKII activity by enhancing sarcolemmal L-type Ca$^{2+}$ currents ($I_{Ca}$), SR Ca$^{2+}$ cycling, and intracellular Ca$^{2+}$ transients (26–28). This mechanism appears to be minimized in the absence of intracellular Ca$^{2+}$ transients, as evidenced by the insensitivity of Thr$^{17}$ phosphorylation to $\beta$-adrenergic stimulation in quiescent myocytes (Fig. 1). In addition to the Ca$^{2+}$-dependent cross-talk, PKA may also enhance CaMKII signaling by inhibiting protein phosphatase 1 (PP1), which is the major phosphatase dephosphorylating PLB (29), either by direct inhibition of PP1 activity via phosphorylation of its regulatory subunit (29) or by indirect inhibition of the phosphatase via phosphorylation and subsequent activation of endogenous phosphatase inhibitor 1 (30, 31). Both mechanisms prevent PP1 from dephosphorylating PLB. In this regard, it has been shown that in beating hearts, a protein phosphatase inhibitor, okadaic acid, permits Thr$^{17}$ PLB phosphorylation in response to high extracellular [Ca$^{2+}$], in the absence of $\beta$-adrenergic stimulation (16). Regardless of the specific mechanism, the potent effect of $\beta$-adrenergic stimulation to enhance CaMKII signaling may masquerade as a sequential phosphorylation of Ser$^{16}$ and Thr$^{17}$ under certain experimental conditions.

**Unique Property of Thr$^{17}$ Phosphorylation: A Frequency Detector**—The present results indicate that CaMKII-mediated phosphorylation of PLB at Thr$^{17}$ is linearly correlated to the increase in stimulation frequency. The strong frequency dependence of Thr$^{17}$ phosphorylation may be attributed largely to an intrinsic “memory” ability of CaMKII. Recent studies have demonstrated that CaMKII undergoes autophosphorylation during activation (13). The autophosphorylated CaMKII retains some kinase activity even in the interval of intracellular Ca$^{2+}$ transients, leading to an accumulative CaMKII activation during high frequency stimulation (13).

The intrinsic memory property of CaMKII makes Thr$^{17}$ PLB phosphorylation unique as compared with PKA-mediated Ser$^{16}$ phosphorylation. As a result of frequency-dependent CaMKII activation, phosphorylation of Thr$^{17}$ PLB may function as a frequency detector of heart rate, the higher the heart rate and the more frequent intracellular Ca$^{2+}$ transients, the greater is CaMKII-mediated Thr$^{17}$ PLB phosphorylation. Because frequency-dependent Thr$^{17}$ PLB phosphorylation is closely associated with the frequency-dependent acceleration of relaxation (Fig. 4), CaMKII-mediated Thr$^{17}$ PLB phosphorylation may provide a constant beat-to-beat intrinsic regulation of cardiac contractile kinetics.

In summary, the present results provide the first documentation that in intact cardiac myocytes, frequency-dependent Thr$^{17}$ PLB phosphorylation by CaMKII occurs independently of PKA-mediated Ser$^{16}$ phosphorylation. The increase in phosphorylation of Thr$^{17}$ PLB is associated with a profound relaxation effect. These conclusions not only challenge the traditional

---

2 R.-P. Xiao, D. Hagemann, and W. Zhu, unpublished data.
sequential model of PLB phosphorylation but also suggest a novel autoregulatory mechanism in cardiac beat-to-beat functional modulation.

Acknowledgments—We thank Drs. Edward G. Lakatta and Ying-Ying Zhou for stimulating discussions and Dr. Harold Spurgeon and Bruce Ziman for their excellent technical support.

REFERENCES
1. Simmerman, H. K. B., and Jones, L. R. (1998) *Physiol. Rev.* 78, 921–947
2. Inui, M., Chamberlin, B. H., Saito, A., and Fleischer, S. (1986) *J. Biol. Chem.* 261, 13333–13341
3. MacDougall, L. K., Jones, L. R., and Cohen P (1991) *Eur. J. Biochem.* 203, 507–516
4. Gupta, R. C., Neumann, J., Watanabe, A. M., Lesch, M., and Sabbah, H. N. (1996) *Am. J. Physiol.* 270, H1159–H1164
5. Luo, W., Chu, G., Sato, Y., Zhou, Z., Kadambi, V. J., and Kranias, E. G. (1998) *J. Biol. Chem.* 273, 4734–4739
6. Drago, G. A., and Colyer, J. (1994) *J. Biol. Chem.* 269, 25073–25077
7. Mayer, E. J., Huckle, W., Johnson, R. G., and McKenna, E. (2000) *Biochem. Biophys. Res. Commun.* 267, 40–48
8. Bean, B. P., Nowycky, M. C., and Tsien, R. W. (1984) *Nature* 307, 371–375
9. Xu, A., Hawkins, C., and Narayanan, N. (1997) *J. Mol. Cell. Cardiol.* 29, 405–416
10. Xiao, R.-P., and Lakatta, E. G. (1993) *Am. J. Physiol.* 264, H1159–H1164
11. MacDougall, L. K., Jones, L. R., and Cohen P (1991) *Eur. J. Biochem.* 203, 507–516
12. Gupta, R. C., Neumann, J., Watanabe, A. M., Lesch, M., and Sabbah, H. N. (1996) *Am. J. Physiol.* 270, H1159–H1164
13. Braun, A. P., Schulman, H. (1995) *Ann. Rev. Physiol.* 57, 417–445
14. Lindemann, J. P., and Watanabe, A. M. (1985) *J. Biol. Chem.* 260, 4516–4525
15. Napolitano, R., Vittone, L., Mundina, C., Chiappe de Cingolani, G., and Mattiazzi, A. (1992) *J. Mol. Cell. Cardiol.* 24, 387–396
16. Mundina-Weilenmann, C. M. DE, Vittone, L., Ortape, M. de Cingolani, G. C., and Mattiazzi, A. (1996) *J. Biol. Chem.* 271, 33561–33567
17. Weilenmann, C. M. DE, Vittone, L., and Mattiazzi, A. (1987) *Basic Res. Cardiol.* 82, 507–516
18. Vittone, L., Mundina, C., Chiappe de Cingolani, G., and Mattiazzi, A. (1993) *Am. J. Physiol.* 258, H1159–H1164