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ORIGINAL ARTICLE

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The relative relevance of phosphorylation of the Thr^{17} residue of phospholamban is different at different levels of β-adrenergic stimulation

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Abstract Contractility and relaxation measurements were combined with the determination of total phospholamban (PLB) phosphorylation and the immunodetection of PLB-phosphorylation sites in the intact, beating rat heart to identify the contributions of PLB phosphorylation at the Thr^{17} and Ser^{16} residues at different levels of β-adrenoceptor stimulation. Whereas with 30–300 nM isoproterenol, phosphorylation of Thr^{17}, the Ca^{2+}-calmodulin-dependent protein kinase-II (CaMKII) site and Ser^{16}, the protein kinase A (PKA) site, contributed approximately 50% each to PLB phosphorylation, and both participated in the relaxant action of isoproterenol, at lower a level of β-adrenoceptor stimulation (isoproterenol 0.3–3 nM), both effects were exclusively due to Ser^{16} phosphorylation. Increasing $[Ca]_o$ at 3 nM isoproterenol, to obtain an increase in contractility comparable to that produced by 30 nM isoproterenol, significantly increased Thr^{17} phosphorylation and the relaxant effect produced by 3 nM isoproterenol. An increase in Thr^{17} phosphorylation and in the relaxant effect of 3 nM isoproterenol was also obtained by phosphatase inhibition (okadaic acid). In this case, Ser^{16} phosphorylation was also increased. Moreover, perfusion with 30 nM isoproterenol in the presence of the PKA inhibitor H-89 decreased phosphorylation at both PLB residues and diminished the inotropic and relaxant responses to the β-agonist. The relative contribution of Thr^{17} phosphorylation to the isoproterenol-induced phosphorylation of PLB and relaxation thus increased with the level of β-adrenoceptor stimulation and the consequent increase in PKA activity. The lack of Thr^{17} phosphorylation at low isoproterenol concentrations might therefore be attributed to a level of PKA activity insufficient to increase $[Ca]_i$ to activate the CaMKII system and/or to inhibit the phosphatase that dephosphorylates PLB

Keywords Phospholamban phosphorylation sites · β-Adrenoceptor stimulation · Myocardial relaxation

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Introduction

Phospholamban (PLB) is a phosphorylatable protein component of the cardiac sarcoplasmic reticulum (SR) that reversibly inhibits the activity of the SR Ca^{2+} pump and SR Ca^{2+} transport. Phosphorylation of PLB relieves this inhibition [10, 16, 17, 18]. Studies on mice with *PLB* gene ablation have demonstrated that PLB is a prominent regulator of basal myocardial contractility [12]. These studies have shown also that phosphorylation of PLB is the main mediator of the contractile and relaxant effects of β-agonists [12]. β-Adrenoceptor-induced phosphorylation of PLB occurs at two different sites: on Ser^{16} by cAMP-dependent protein kinase (PKA) and on Thr^{17} by Ca^{2+}-calmodulin-dependent protein kinase-II (CaMKII) [15, 23]. However, the relative contribution of each phosphorylation site to total PLB phosphorylation and the mechanical effects of β-adrenoceptor agonists, remains controversial. While our studies have indicated that each phosphorylation site contributes to approximately 50% of the total phosphorylation of PLB and of the relaxant effect produced by a maximal isoproterenol concentration [15, 19, 20], other studies have suggested that the phosphorylation of Thr^{17} plays only a minor physiological role in the inotropic and relaxant effects of β-adrenergic stimulation [4, 11]. Experiments in transgenic mice have indicated further that Ser^{16} phosphorylation is sufficient for mediating the maximal cardiac response to β-agonists [6].

It has also been shown that during β-adrenergic stimulation, phosphorylation of Ser^{16} appears to be a prerequisite for the phosphorylation of Thr^{17} residue [13, 19]. However, an increase in Thr^{17} phosphorylation can occur in the absence of an enhancement of Ser^{16}

phosphorylation in intact hearts. This phosphorylation is associated with relaxation and can be detected only when extracellular Ca^{2+} is increased to activate CaMKII and phosphatases are inhibited [15, 21]. Recent experiments in isolated myocytes have shown a frequency-dependent increase in Thr^{17} phosphorylation in the absence of Ser^{16} phosphorylation together with an enhanced relaxation [8]. Finally, experiments from our laboratory have shown a striking increase in the phosphorylation of Thr^{17} at the beginning of reperfusion after a period of global ischaemia. Again, this increase occurred in the absence of any significant change in Ser^{16} phosphorylation and contributed to the recovery of relaxation at the onset of reperfusion [22]. Taken together, these results indicate that the relevance of Thr^{17} phosphorylation as an independent regulator of myocardial relaxation and as a possible major player in the relaxant effect of β-adrenoceptor stimulation, should be reconsidered.

The present experiments combined contractility and relaxation measurements in the intact beating heart with the determination of total PLB phosphorylation by ^{32}P incorporation and the immunodetection of PLB-phosphorylation sites, with the aim of establishing the relative contribution of each phosphorylation site to the total PLB phosphorylation and to the relaxant effect of β-adrenoceptor stimulation at different isoproterenol concentrations. The evidence to be presented provides a clue for the interpretation of previous contradictory findings.

Materials and methods

Heart perfusions

Experiments were performed on isolated hearts from male Wistar rats (250–350 g) perfused according to the Langendorff technique as previously described [15, 19]. The composition of the physiological salt solution (PSS) was (in mM): 128.3 NaCl, 4.7 KCl, 1.35 $CaCl_2$, 20.2 $NaHCO_3$, 0.4 NaH_2PO_4, 1.1 $MgCl_2$, 11.1 glucose and 0.04 Na_2EDTA. Unless otherwise specified, this solution was equilibrated with 95% O_2/5% CO_2 to give a pH of 7.4. The mechanical activity of the heart was assessed by passing a latex balloon connected to a pressure transducer (Namic, perceptor DT disposable transducer) into the left ventricle. The balloon was filled with aqueous solution to achieve a left ventricular end-diastolic pressure of 8–14 mmHg. Hearts were perfused with PSS for 10–15 min for stabilization and then during the next 4 min with different interventions, as described in the Results. Isovolumetric pressure and its first derivative (dP/dt) were recorded on a two-channel pen recorder (Gould model 2200S) fitted with a transducer amplifier (Gould model 13-4615-50) and a differentiating amplifier (Gould model 13-4615-71). To assess ^{32}P incorporation into PLB, hearts were perfused for 60 min with recirculating PSS containing 10 μm Ci/ml $^{32}P_i$ and then subjected to the different interventions as described in the Results. At the end of the experimental period, the ventricles were freeze-clamped, pulverized and stored at –70°°C until biochemical assay.

Preparation of SR membrane vesicles

SR membrane vesicles were isolated under conditions that prevent dephosphorylation, as previously described [15, 21] from the pulverized ventricular tissue of hearts subjected to the different treatments. Protein was measured by the method of Bradford using bovine serum albumin as standard. The yield was 1–2 mg membrane vesicles protein/g cardiac tissue.

Electrophoresis and Western blot analysis

SDS-PAGE was performed using 10% acrylamide slab gels as previously described [15, 21]. For immunological detection of PLB phosphorylation sites, 15 μg SR membrane protein was electrophoresed in mini gels. This amount of protein is within the range of linearity of the relationship between signal intensity of the site-specific phosphorylated PLB and the amount of protein loaded per gel lane (see Results). Proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore) and probed with polyclonal antibodies raised to a phospholamban peptide (residues 9–19) phosphorylated at Ser^{16} or at Thr^{17} (1:5,000) (Cyclacel, UK). Immunoreactivity was visualized by peroxidase-conjugated antibodies using a peroxidase-based chemiluminescence detection kit (Boehringer Mannheim, Germany). The signal intensity of the bands was quantified by optical densitometric analysis and expressed as percentages of the phosphorylation of Ser^{16} and Thr^{17} induced by 30 nM isoproterenol, a concentration that produces maximal phosphorylation of PLB residues and mechanical response [15], run in parallel with each experimental series. To assess ^{32}P incorporation into PLB, 300 μg membrane protein was electrophoresed per gel lane. Gels were run in duplicate: one for autoradiography, the other for liquid-scintillation counting. Quantitative results were expressed as picomoles ^{32}P incorporated into PLB per milligram SR protein based on the specific activity of ^{32}P in phosphocreatine [1].

Phosphatase activity

Phosphatase activity, measured by the release of ^{32}P from ^{32}P-labelled phosphorylase *a*, was assayed in membranes isolated from hearts perfused with non-radioactive solution, as previously described [14].

Statistics

Data are expressed as means±SEM of *n* preparations. Student's *t*-test for unpaired observations was used to establish the significance of differences between means. $P<0.05$ was considered significant.

Results

Relative contribution of phosphorylation of Ser^{16} and Thr^{17} residues to isoproterenol-induced phosphorylation of PLB

Figure 1 shows the effect of increasing the isoproterenol concentration from 0.3 to 300 nM on ^{32}P incorporation into PLB at normal extracellular $[Ca^{2+}]$ ($[Ca]_o$ 1.35 mM) and under conditions chosen to prevent Ca^{2+} influx to the cardiac cells (0.07 mM $[Ca]_o$ plus 0.4 μM nifedipine). The virtual suppression of Ca^{2+} influx significantly decreased total PLB phosphorylation by about 50% at isoproterenol concentrations of 10–300 nM, but did not affect PLB phosphorylation at low isoproterenol concentrations (0.3 and 3 nM). Figure 2 depicts the results of the immunodetection of site-specific phosphorylated PLB. Figure 2A shows the linear correlation between each antibody reaction and the amount of SR membrane protein loaded

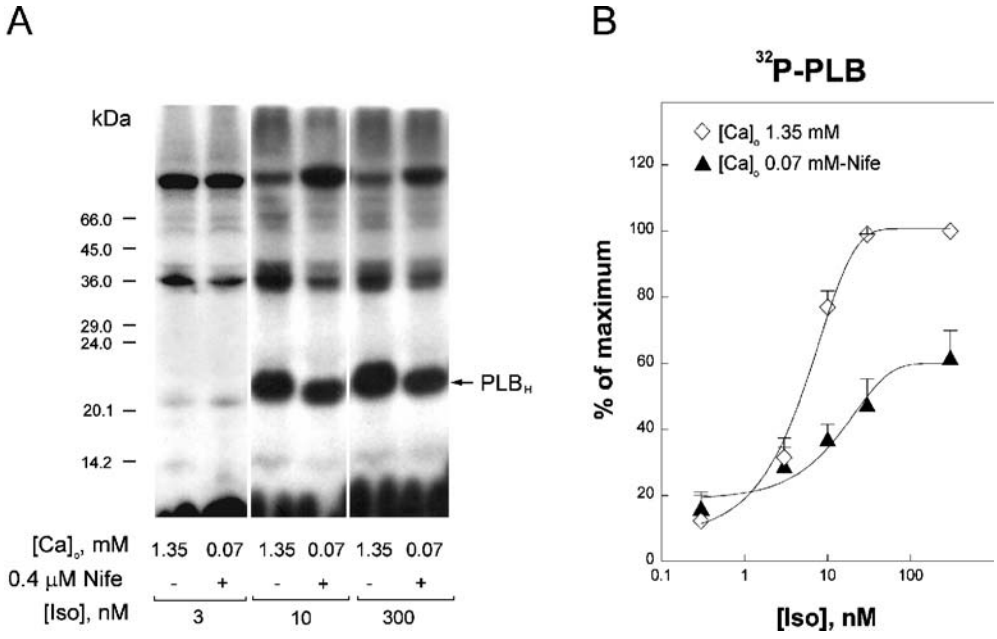


Fig. 1A, B Isoproterenol concentration-dependent increase in ^{32}P incorporation into phospholamban (*PLB*), at two different levels of extracellular [Ca^{2+}] ([Ca]$_o$). **A** Autoradiograph of SR membrane vesicles isolated from rat hearts perfused with ^{32}P and then with different isoproterenol concentrations at different [Ca]$_o$. **B** Summary of experiments as shown in **A**. Results are expressed as mean (±SEM) percentage of maximal ^{32}P incorporation into PLB, n=4–8 (*Iso* isoproterenol, *Nife* nifedipine, *PLB$_H$* high-molecular-weight form of PLB)

per gel lane within the range 5–60 μg. The calibration curves for both antibodies (P-Ser^{16}-PLB and P-Thr^{17}-PLB) were highly reproducible in all experiments with correlation coefficients (r) of 0.99. Figure 2B shows the results obtained from hearts perfused with isoproterenol in the presence of 0.07 mM [Ca]$_o$ plus nifedipine. Under these conditions the isoproterenol-induced increase in phosphorylation of PLB was exclusively due to the increase in Ser^{16} phosphorylation. Phosphorylation of Thr^{17} remained at basal levels at all isoproterenol concentrations, in agreement with previous findings [15]. Phosphorylation of Ser^{16} and Thr^{17} residues at different isoproterenol concentrations at 1.35 mM [Ca]$_o$ is compared in Fig. 2C. Phosphorylation of the Thr^{17} residue was shifted to higher isoproterenol concentrations with respect to phosphorylation of Ser^{16}. The half-maximal isoproterenol concentration (EC_{50}) was 8.37±1.80 nM for Ser^{16} phosphorylation and 17.02±3.35 nM for Thr^{17} phosphorylation (P<0.05). A shift in the same direction has been described previously in the intact heart and isolated neonatal myocytes [2, 11]. As evident from Fig. 2C, phosphorylation of Ser^{16} was higher than basal values at all isoproterenol concentrations tested, while the phosphorylation of Thr^{17} increased above basal levels from 10 nM isoproterenol. Taken together, the results support the conclusion that, both phosphorylation sites contribute to approximately the same extent to the increase in total PLB phosphorylation produced by maximal β-adrenoceptor stimulation. At low isoproterenol concentrations, the increase in PLB phosphorylation appears to be exclusively determined by the increase in the phosphorylation of Ser^{16} residue.

Relative contribution of the phosphorylation of Ser^{16} and Thr^{17} residues of PLB to the isoproterenol-induced relaxant effect

According to the results shown in Figs. 1 and 2, the contribution of Thr^{17} phosphorylation to the relaxant effect of isoproterenol should increase with increasing isoproterenol concentration. To test this hypothesis, we analysed the effect of inhibiting the CaMKII pathway on total PLB phosphorylation, PLB phosphorylation sites, relaxation (half relaxation time, $t_{1/2}$) and contractility (the rate of rise of left ventricular pressure +dP/dt), at three different levels of β-adrenergic stimulation: 30, 10 and 3 nM isoproterenol (Figs. 3, 4 and 5). At these isoproterenol concentrations, total PLB phosphorylation reached approximately 100, 75 and 30% of maximum, respectively. Inhibition of the CaMKII pathway was achieved by either the calmodulin antagonist W-7 or decreasing [Ca]$_o$. Whereas W-7 did not affect the positive inotropic effect of isoproterenol, the decrease in [Ca]$_o$ was chosen to cancel the positive inotropic effect of each isoproterenol concentration and to return contractility to control levels (see Fig. 5B). Figure 3A shows the overall results of ^{32}P incorporation into PLB from hearts perfused with 30 nM isoproterenol at 1.35 mM [Ca]$_o$, at 1.35 mM [Ca]$_o$ plus 1 μM W-7 and at low [Ca]$_o$ (0.25 mM). The presence of either W-7 or low [Ca]$_o$ significantly reduced the isoproterenol-induced PLB phosphorylation by 20.6±8% and 33.6±3.5% respectively. Immunodetection of site-specific phosphorylated PLB indicated that this reduction was exclusively due to a decrease in the phosphorylation of Thr^{17} residue, without any significant change in Ser^{16} phosphorylation (Fig. 3B). The overall results of this group of experiments and of the experiments performed at 10 and 3 nM isoproterenol are depicted in Figs. 4 and 5. At 30 nM isoproterenol, perfusion with W-7 or with

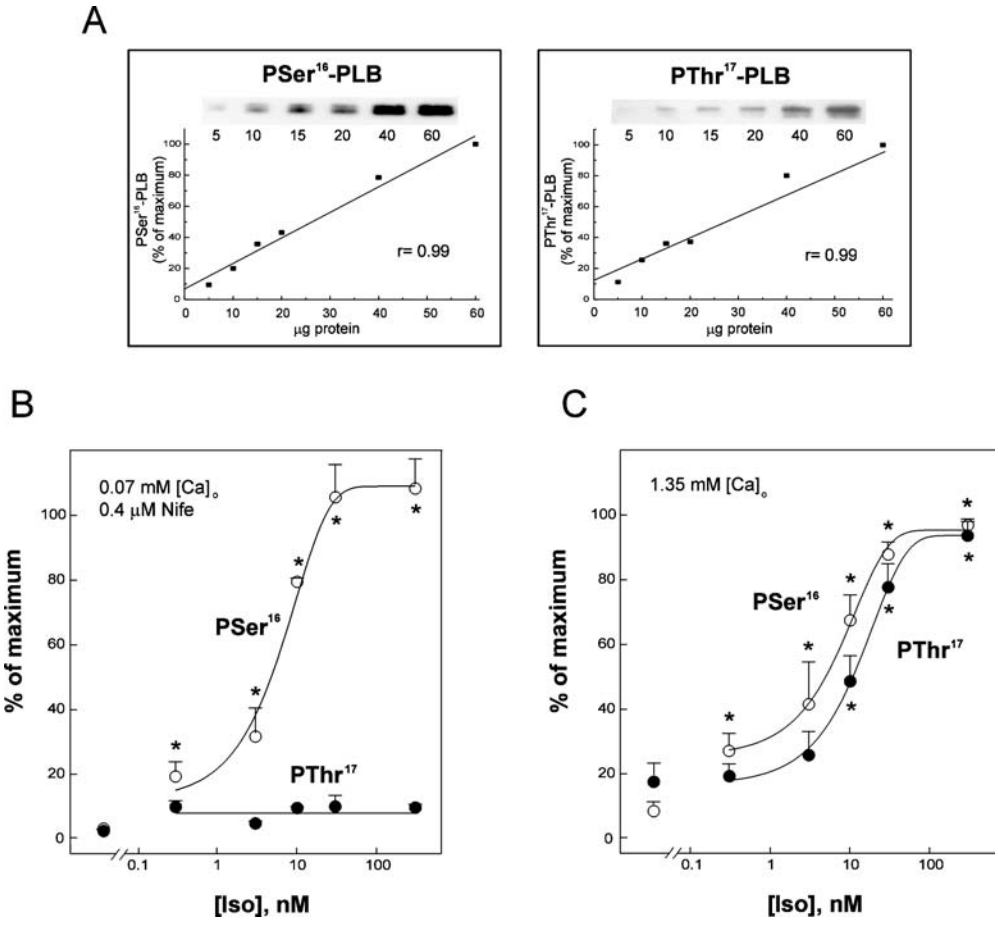


Fig. 2A–C Isoproterenol concentration-dependent increase in the phosphorylation of Ser^{16} and Thr^{17} residues at two different levels of $[Ca]_o$. **A** Linear correlation between the antibody reaction and the amount of sarcoplasmic reticulum (SR) membrane protein loaded per gel lane in the range of 5–60 µg. SR membrane protein was isolated from a heart perfused with 30 nM isoproterenol, a concentration that produced maximal Ser^{16} and Thr^{17} phosphorylations. Immunoblots and data from one experimental run. **B** The decrease in $[Ca]_o$ as in Fig. 1, did not significantly affect the phosphorylation of Ser^{16} but inhibited the increase in the phosphorylation of Thr^{17} at all isoproterenol concentrations. **C** At control $[Ca]_o$ levels, the increase in the phosphorylation of Thr^{17} was shifted to the right relative to the increase in the phosphorylation of Ser^{16}. Results are expressed as percentage of the maximal signal achieved in each experimental series. In **B** and **C**, mean±SEM values obtained after densitometric analysis of the signal from between three and ten immunoblots. $*P<0.05$ vs. control values at low $[Ca]_o$ (**B**) and normal $[Ca]_o$ (**C**)

0.25 mM $[Ca]_o$ produced a decrease in Thr^{17} phosphorylation that was about twice the decrease in total PLB phosphorylation (Fig. 4). This is in agreement with an approximately 50% contribution of Thr^{17} phosphorylation to the total PLB phosphorylation at this high isoproterenol concentration and normal $[Ca]_o$. The decrease in Thr^{17} phosphorylation in the presence of W-7 or low $[Ca]_o$ was associated with a significant reduction of the relaxant effect of the β-agonist, i.e. the decrease in $t_{1/2}$ was attenuated from –18.8±2.5 ms (Iso) to –12.5±1.8 ms and –13.2±1.8 ms respectively (Fig. 5A). At 10 nM isoproterenol the decrease in $[Ca]_o$ significantly diminished the phosphorylation of the Thr^{17} residue without affecting the phosphorylation of the Ser^{16} residue (Fig. 4), whereas the isoproterenol-induced decrease in $t_{1/2}$ was attenuated significantly from –14.8±0.5 ms to –11.4±1.2 ms (Fig. 5A). At 3 nM isoproterenol decreasing $[Ca]_o$ had no significant effect on the phosphorylation of either Thr^{17} or Ser^{16} residues (Fig. 4), nor did it modify the relaxant effect of isoproterenol (Fig. 5A).

What are the prerequisites for the phosphorylation of Thr^{17} in the intact heart?

Experiments in transgenic mice in which Ser^{16} was mutated to Ala have demonstrated convincingly that Ser^{16} phosphorylation is a prerequisite for the phosphorylation of Thr^{17} [13]. On the other hand, our previous experiments have shown a significant increase in Thr^{17} phosphorylation by increasing $[Ca]_o$ to activate CaMKII and inhibiting the major phosphatase that dephosphorylates PLB (protein phosphatase-1, PP1) by either 1 µM okadaic acid [15] or by acidic pH [21]. Although in these experiments there was no significant increase in Ser^{16} phosphorylation, basal levels of Ser^{16} phosphorylation

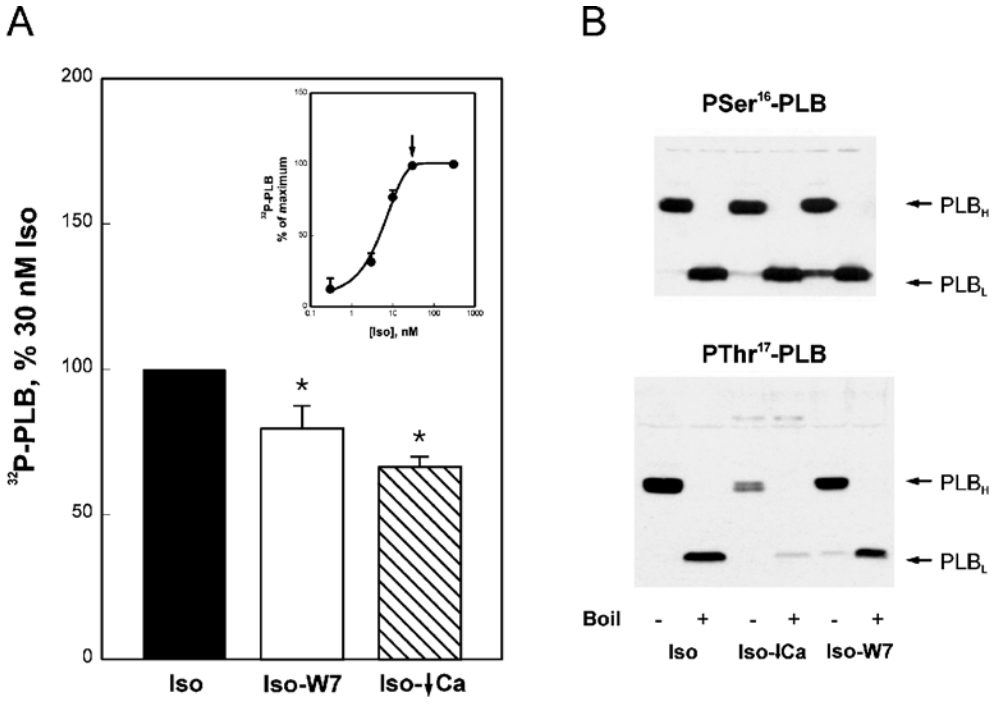
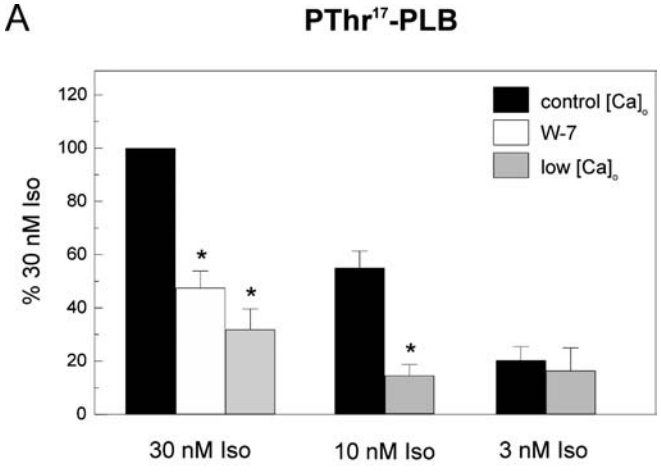


Fig. 3 **A** Effect of W-7 and of decreasing $[Ca]_o$ on isoproterenol-induced ^{32}P incorporation into PLB. Perfusion with 30 nM isoproterenol (*Iso*) in the presence of 1 μM W-7 (*Iso-W7*) and with isoproterenol at 0.25 mM $[Ca]_o$ (*Iso-↓Ca*). The *inset* in the figure indicates that the effect of W-7 and low $[Ca]_o$ was studied at the "plateau" of the concentration/response curve to isoproterenol. Mean±SEM; n=11, 6 and 5 experiments (for Iso, Iso-W7 and Iso-↓Ca respectively). *P<0.05 vs. Iso. **B** Effect of W-7 and of decreasing $[Ca]_o$ on the phosphorylation of Ser^{16} and Thr^{17} residues of PLB. Immunoblots of SR membrane vesicles isolated from hearts perfused as in **A** (PLB_L low-molecular-weight form of PLB)

might have been sufficient to allow phosphorylation of Thr^{17}. To test this possibility, we first selected from one of the above groups (high $[Ca]_o$ plus okadaic acid), those experiments in which basal phosphorylation of Ser^{16} was not detectable. A significant increase in Thr^{17} phosphorylation was nevertheless observed (Fig. 6). These results imply that phosphorylation of the Thr^{17} residue of PLB might occur in the intact heart even in the absence of detectable basal levels of Ser^{16} phosphorylation. As a further test, a new series of experiments was performed in the presence of 1 μM H-89, a PKA inhibitor, in an attempt to avoid completely basal phosphorylation of Ser^{16}. This concentration of H-89 is some 20-fold higher than the inhibitory constant for PKA, but it is still far below that for other protein kinases [5]. In these experiments, $[Ca]_o$ was increased to 3.85 mM to activate the CaMKII-dependent pathway and pH_o was decreased from 7.40 to 6.85 by increasing the CO_2 of the gas mixture from 5% to 20% to inhibit PP1. In isolated rat myocytes the same increase in CO_2 decreases pH_i from 7.18±0.06 to 6.73±0.04 [21]. This pH_i inhibits PP1 by approximately 30% [14]. Thr^{17} phosphorylation was significantly increased at high $[Ca]_o$ under acidic conditions in both the absence or presence of H-89, in spite of the fact that in this latter situation, phosphorylation of Ser^{16} was virtually absent (Fig. 6). These results support the idea that phosphorylation of the Ser^{16} residue of PLB is not per se a prerequisite for phosphorylation of the Thr^{17} residue. However under β-adrenoceptor stimulation, phosphorylation of Ser^{16} could be a prerequisite for the increase in intracellular Ca^{2+} to a sufficient level to activate the CaMKII-dependent pathway of PLB phosphorylation. Alternatively, since PP1 is inhibited by PKA-dependent phosphorylation [7], another prerequisite for phosphorylation of the Thr^{17} residue may be a given level of phosphatase inhibition. To test these possibilities different types of experiments were performed. In the presence of 3 nM isoproterenol: (1) $[Ca]_o$ was increased to 2.5 mM in an attempt to activate CaMKII; (2) 0.6 μM okadaic acid was administered to inhibit PLB phosphatase. Control experiments in vitro showed that this concentration of okadaic acid decreases PP1 activity by 97.2±1%. Both the increase in $[Ca]_o$ and the concentration of okadaic acid were selected on the basis of preliminary experiments to produce an increase in contractility similar to that evoked by 30 nM isoproterenol. Figure 7 shows the results of these experiments. Increasing $[Ca]_o$ at 3 nM isoproterenol increased the phosphorylation of Thr^{17} by approximately 30%. This increase was associated with a significant reduction of $t_{1/2}$ and occurred in the absence of significant changes in the phosphorylation of Ser^{16}. In the presence of 3 nM isoproterenol, okadaic acid produced an increase in Thr^{17} phosphorylation higher than that produced by high $[Ca]_o$ and similar to that produced by 30 nM isoproterenol. Okadaic acid also increased Ser^{16} phosphorylation and decreased $t_{1/2}$ to a level similar to that elicited by 30 nM isoproterenol. Thus, the lack of increase of Thr^{17} phosphorylation at 3 and 0.3 nM isoproterenol may be explained by either too small an increase in $[Ca]_i$ to activate CaMKII and/or a low level of PLB phosphatase inhibition as a consequence of low PKA activation. Since PKA activation seems to be a prerequisite for the isoproterenol-induced increase not only for Ser^{16} but also for Thr^{17} phosphorylation, the inhibition of PKA should decrease the phosphorylation of both PLB residues. Immunoblots of a typical experiment showed in Fig. 8 demonstrated that this is in fact the case. The overall results of these experiments, depicted in the same figure,



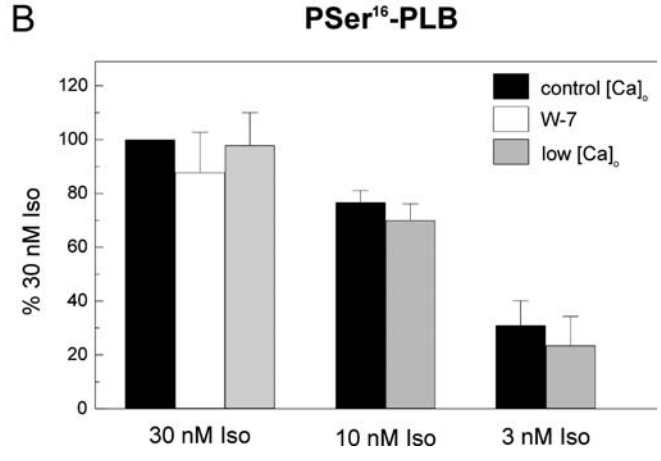
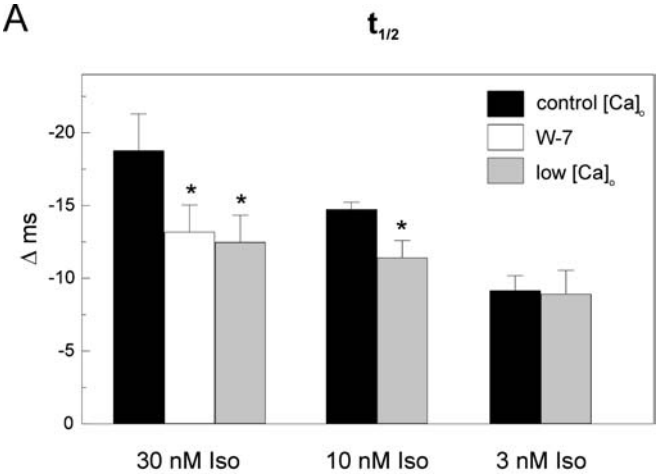


Fig. 4 Effect of W-7 and of decreasing $[Ca]_o$ on PLB phosphorylation sites [$PThr^{17}$-PLB (**A**) and $PSer^{16}$-PLB (**B**)] at different isoproterenol concentrations. Mean±SEM; n=5–10 experiments; *P<0.05 vs. corresponding Iso concentration at control $[Ca]_o$



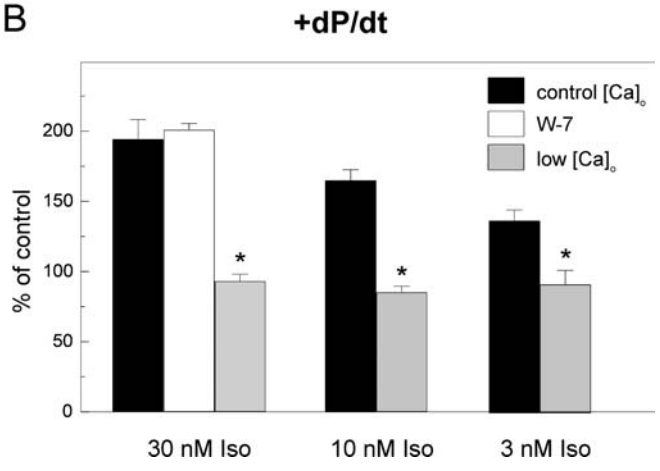


Fig. 5A, B Effect of W-7 and of decreasing $[Ca]_o$ on half relaxation time ($t_{1/2}$, **A**, Δ *ms*: difference from control values) and contactility (maximal rate of rise of pressure, +*dP/dt*, **B**) at different isoproterenol concentrations. Mean±SEM, n=5–10 experiments. *P<0.05 vs. corresponding Iso concentration at control $[Ca]_o$

show that perfusion with H-89 in the presence of 30 nM isoproterenol decreased the phosphorylation of Ser^{16} and Thr^{17} residues of PLB and the positive inotropic and the relaxant effects of isoproterenol. The decrease in PLB phosphorylation residues was significantly greater for the Thr^{17} site, in line with a shift to the right of the Thr^{17} phosphorylation-isoproterenol relationship (Fig. 2C).

Discussion

Previous experiments from our laboratory have indicated that the phosphorylation of the Thr^{17} residue of PLB contributes to approximately 50% to the phosphorylation of PLB produced by a maximal isoproterenol concentration [15]. The present study extends our previous findings by showing firstly that the contribution of each phosphorylation site to the total phosphorylation of PLB and to the relaxant effect produced by β-adrenoceptor stimulation is different at different isoproterenol concentrations. The relative contribution of Thr^{17} phosphorylation to these isoproterenol effects increased with the increase in the level of β-adrenoceptor stimulation. Secondly, at low isoproterenol concentrations, phosphorylation of Thr^{17} could be observed only by increasing intracellular Ca^{2+} to activate CaMKII and/or inhibiting PP1, the phosphatase that dephosphorylates PLB. Thirdly, a level of PKA activation higher than that produced by 3 nM isoproterenol appears to be the prerequisite for the isoproterenol-induced phosphorylation of Thr^{17} of PLB.

Different investigators have addressed the relative contribution of Thr^{17} and Ser^{16} to the total phosphorylation of PLB and to the relaxant effect of β-adrenoceptor stimulation [2, 4, 6, 11, 13, 15]. Most of the in vivo experiments have suggested that the phosphorylation of Ser^{16} prevails over that of Thr^{17} in determining the functional effects of β-adrenoceptor stimulation [4, 6, 11,

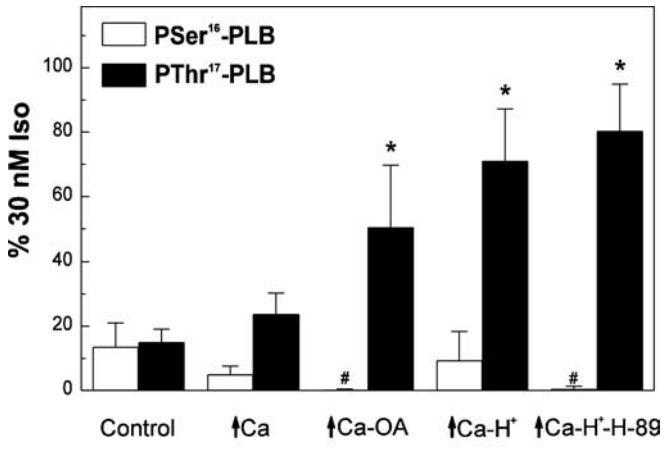


Fig. 6 Effect of increasing $[Ca]_o$ to 3.85 mM (↑*Ca*) in the presence of phosphatase inhibition [1 μM okadaic acid (↑*Ca-OA*) or acidosis (↑*Ca-H*$^+$)] or with the protein kinase A (PKA) inhibitor H-89 and acidosis (↑*Ca-H*$^+$*-H-89*) on phosphorylation of PLB residues. Mean±SEM, *n*=3–13 experiments. *, $^{\#}P<0.05$ vs. control for Thr^{17} and Ser^{16} respectively

13]. Other lines of evidence indicate, however, that phosphorylation of Thr^{17} may play an independent and non-negligible role in the regulation of myocardial relaxation, both in the presence and in the absence of β-adrenoceptor stimulation [3, 8, 15, 21, 22]. The present findings may give a clue for the interpretation of these apparently contradictory conclusions, in that, at high isoproterenol concentrations (30–300 nM), the phosphorylation of Thr^{17} is responsible for approximately 50% of the total phosphorylation of PLB. The results further show that phosphorylation of Thr^{17} also participates in the isoproterenol-induced relaxant effect, as suggested by the close association between the decrease in Thr^{17} phosphorylation and the decrease in the relaxant effect of isoproterenol depicted in Figs. 4 and 5. These observations argue for a significant role of Thr^{17} phosphorylation at high levels of β-adrenoceptor stimulation. The contribution of Thr^{17} phosphorylation to the total PLB phosphorylation and to the relaxant effect of isoproterenol was still evident at intermediate isoproterenol concentrations (10 nM, Figs. 1, 2, 4 and 5). In contrast, at low isoproterenol concentrations (0.3–3 nM), inhibition of the CaMKII cascade did not significantly affect either the isoproterenol-induced PLB phosphorylation or the relaxant effect of the β-agonist, which would indicate that the increase in PLB phosphorylation was due only to the increase in phosphorylation of the Ser^{16} residue (Figs. 1, 2, 4 and 5). These results are consistent with the shift to the right of the Thr^{17} phosphorylation-isoproterenol relationship compared with the Ser^{16} phosphorylation curve (Fig. 2C). The increase in Thr^{17} phosphorylation and the associated decrease in $t_{1/2}$ that occurred at 3 nM isoproterenol, when either $[Ca]_o$ was increased or the phosphatase inhibitor okadaic acid was present (Fig. 7), support the idea that the lack of Thr^{17} phosphorylation at low isoproterenol concentrations is the consequence of a low activity of CaMKII that phosphorylates Thr^{17} and/or a high activity of the phosphatase that dephosphorylates the residue, such that the net balance would favour the dephosphorylated state of the residue. This balance would

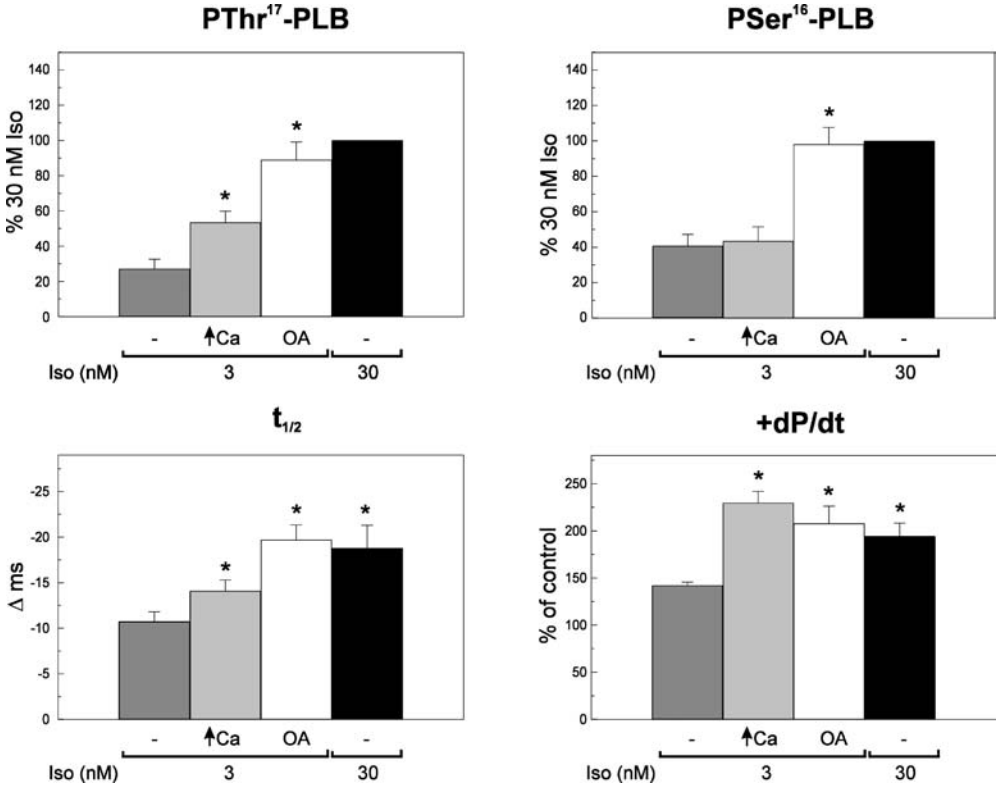


Fig. 7 Effect of increasing $[Ca]_o$ to 2.5 mM (↑*Ca*) or inhibiting phosphatases by the addition of 0.6 μM okadaic acid (OA) at 3 nM isoproterenol on Thr^{17} (*upper left*) and Ser^{16} (*upper right*) phosphorylation, relaxation, $t_{1/2}$ (*lower left*; Δ *ms*: difference from control values) and contractility +dP/dt (*lower right*) Phosphorylation of Ser^{16} and Thr^{17} are expressed as the percentage of the phosphorylation produced by 30 nM Iso (*solid bar*). Mean±SEM, *n*=6–8 experiments. $^*P<0.05$ vs. 3 nM Iso

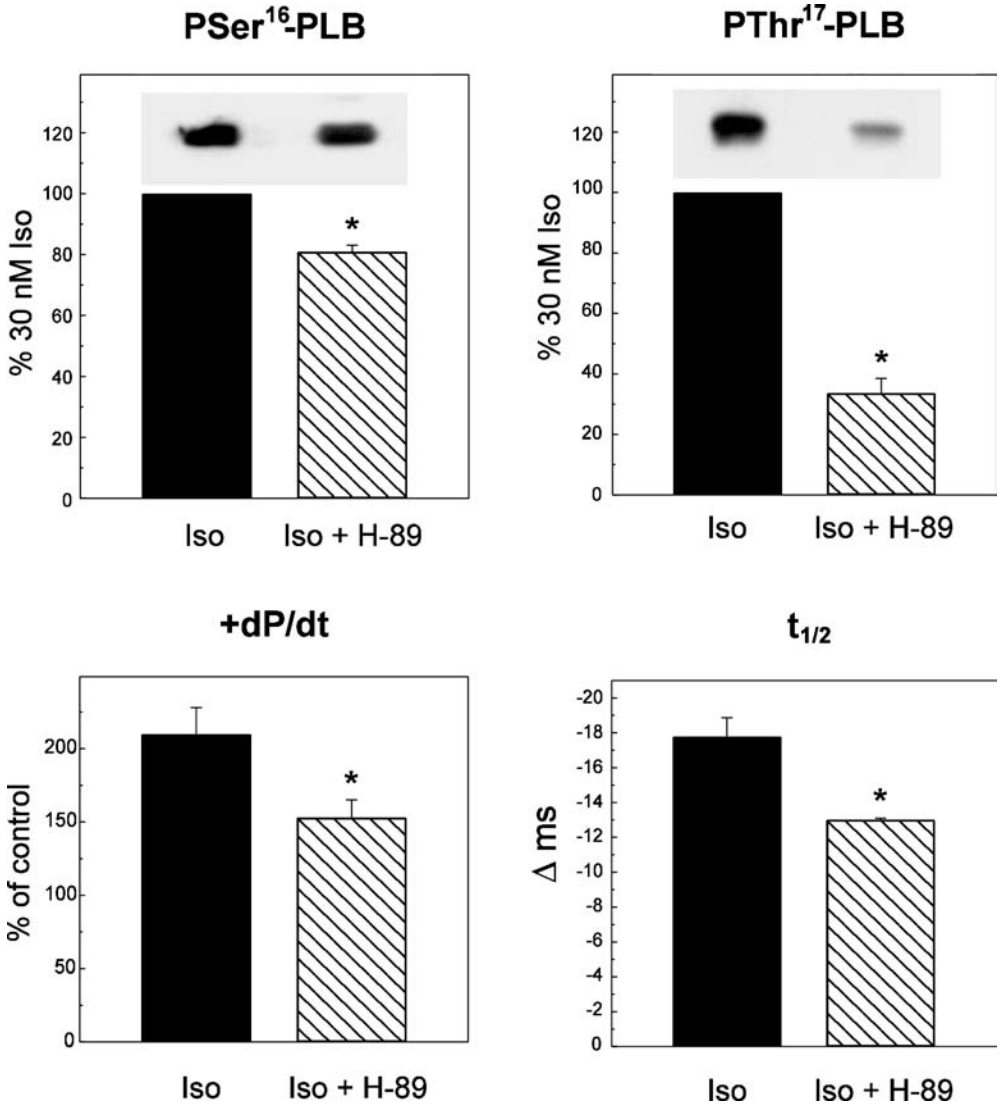


Fig. 8 Effect of PKA inhibition (20 μM H-89; *Iso+H-89*) on Thr^{17} (*upper left*) and Ser^{16} (*upper right*), contractility (+dP/dt, *lower left*) and the relaxant effect ($t_{1/2}$, *lower right*) produced by 30 nM Iso. Means±SEM, n=3–4 experiments. *P<0.05 vs. 30 nM Iso

actually arise from an insufficient PKA activation at these low levels of β-adrenoceptor stimulation. A low degree of PKA activation would fail to produce a sufficient increase in the phosphorylation of both Ser^{16} residue of PLB and Ca^{2+} channels [9], to increase intracellular Ca^{2+} and activate CaMKII, and a sufficient inhibition of PP1 to preclude the dephosphorylation of the Thr^{17} site [7]. Thus, a decrease in PKA activity would decrease not only the phosphorylation of the Ser^{16} residue of PLB but also that of the Thr^{17} site. This prediction was shown to be true in the experiments in which isoproterenol was perfused with the PKA inhibitor H-89 (Fig. 8).

Experiments in isolated myocytes have shown a significant correlation between phosphorylation of Ser^{16} and Thr^{17} and both the contraction amplitude and the rate of relaxation after different concentrations of isoproterenol [4]. However, the correlation was lower for the relationship between Thr^{17} phosphorylation and both contraction amplitude and rate of relaxation, than that between Ser^{16} phosphorylation and these parameters. This lower correlation might be the consequence of the lack of phosphorylation of Thr^{17} at low isoproterenol concentrations described in the present experiments. Furthermore, previous experiments in transgenic mice have indicated that Ser^{16} phosphorylation is a prerequisite for the phosphorylation of Thr^{17} [13]. It was speculated that the lack of Thr^{17} phosphorylation observed in the hearts from mice in which Ser^{16} was mutated to Ala, might have been attributable to the low levels of Ca^{2+} available for activating the CaMKII system [13]. The present experiments addressed this possibility. The lack of phosphorylation of Thr^{17} at low isoproterenol concentrations was changed to a significant increase when $[Ca]_o$ was increased. This increase in $[Ca]_o$ seemed to be sufficient to increase Thr^{17} phosphorylation by approximately 30% without the need for further inhibition of phosphatases, and was associated with enhancement of the relaxant effect of 3 nM isoproterenol. Moreover, an increase in contractility evoked by phosphatase inhibition similar to that induced by increasing $[Ca]_o$, produced an increase in

Thr^{17} phosphorylation that was twice that evoked by increasing $[Ca]_o$. Of note, the increase in the phosphorylation of Thr^{17} in the presence of okadaic acid will result not only from inhibition of PLB phosphatase, but also from the increase in CaMKII activity due to the increase in intracellular Ca^{2+} produced by the phosphorylation of both the Ser^{16} residue of PLB (Fig. 7) and the Ca^{2+} channels [9].

In summary, evidence has been presented indicating that the contribution of Thr^{17} to the total PLB phosphorylation and to the relaxant effect evoked by isoproterenol decreases with the decrease in the level of β-adrenoceptor stimulation. The present results suggest that only the maximal concentrations of isoproterenol provide the conditions that lead to a net balance between the activities of kinases and phosphatases such that the phosphorylation of Thr^{17} residue of PLB is favoured. It is noteworthy that these conditions may not be achieved even at the higher isoproterenol concentrations if the increase in intracellular Ca^{2+} is only modest, such as occurs when extracellular Ca^{2+} is decreased (Fig. 4) or in the transgenic animals in which Ser^{16} is mutated to Ala [13]. The increase in contractility in these transgenic mice at the higher isoproterenol concentrations is similar to that produced by 1 nM isoproterenol in the wild-type animals. If the results of the current experiments can be extrapolated to the mouse heart, then the phosphorylation of Thr^{17} residue should not be expected in the mutant mice in all the range of isoproterenol concentrations explored, as was indeed the case [13].

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