THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 271, No. 52, Issue of December 27, pp. 33561–33567, 1996
Printed in U.S.A.

Immunodetection of Phosphorylation Sites Gives New Insights into the Mechanisms Underlying Phospholamban Phosphorylation in the Intact Heart*

(Received for publication, August 29, 1996, and in revised form, September 26, 1996)

Cecilia Mundiña-Weilenmann‡, Leticia Vittone‡, Manuel Ortale§, Gladys Chiappe de Cingolani‡, and Alicia Mattiazzi‡¶

From the Centro de Investigaciones Cardiovasculares, Facultad de Ciencias Médicas, Universidad Nacional de La Plata, 60 y 120, 1900 La Plata, Argentina

Phosphorylation site-specific antibodies, quantification of ^{32}P incorporation into phospholamban, and simultaneous measurements of mechanical activity were used in Langendorff-perfused rat hearts to provide further insights into the underlying mechanisms of phospholamban phosphorylation. Immunological detection of phospholamban phosphorylation sites showed that the isoproterenol concentration-dependent increase in phospholamban phosphorylation was due to increases in phosphorylation of both Ser^{16} and Thr^{17} residues. When isoproterenol concentration was increased at extremely low Ca^{2+} supply to the myocardium, phosphorylation of Thr^{17} was virtually absent. Under these conditions, ^{32}P incorporation into phospholamban, due to Ser^{16}, decreased by 50%. Changes in Ca^{2+} supply to the myocardium either at constant β-adrenergic stimulation or in the presence of okadaic acid, a phosphatase inhibitor, exclusively modified Thr^{17} phosphorylation. Changes in phospholamban phosphorylation due to either Ser^{16} and/or Thr^{17} were paralleled by changes in myocardial relaxation. The results indicate that cAMP-(Ser^{16}) and Ca^{2+}-calmodulin (Thr^{17})-dependent pathways of phospholamban phosphorylation can occur independently of each other. However, in the absence of β-adrenergic stimulation, phosphorylation of Thr^{17} could only be detected after simultaneous activation of Ca^{2+}-calmodulin-dependent protein kinase and inactivation of phosphatase. It is suggested that under physiological conditions, this requisite is only filled by cAMP-dependent mechanisms.

Cardiac sarcoplasmic reticulum (SR)[1] Mg^{2+}-dependent Ca^{2+}-activated ATPase is regulated by phospholamban, a protein also located in the SR membranes. Phospholamban, which normally associates with the Ca^{2+} pump inhibiting its function, is critically involved in the regulation of cardiac contraction and relaxation. Phosphorylation of phospholamban by either cAMP-dependent protein kinase (PKA) or Ca^{2+}-calmodulin-dependent protein kinase (CaMKII) causes dissociation of phospholamban from the pump, thus increasing ATPase activity and the rate of Ca^{2+} uptake by the SR (1, 2). The increased rate of SR Ca^{2+} uptake enhances the rate of the Ca^{2+} transient decline and increases the Ca^{2+} available for subsequent release, inducing increases in cardiac relaxation and contractility, respectively. *In vitro* experiments indicate that PKA and CaMKII phosphorylate phospholamban at two different sites, Ser^{16} and Thr^{17}, respectively (3). These phosphorylations are independent of each other, and when both are operating, they appear to have an additive action (4). In the intact heart, β-adrenergic stimulation phosphorylates phospholamban at both sites (5), which indicates that PKA- and CaMKII-dependent pathways are also working in the functioning heart. Whether these phosphorylation mechanisms are independent of each other and additive, as described in the isolated SR membranes, remains unknown. Different attempts to phosphorylate phospholamban by CaMKII in the intact heart have systematically failed unless cAMP levels within the cell increase (6–11). This consistent finding strongly suggests an interaction between PKA and CaMKII pathways of phospholamban phosphorylation in the intact heart. The nature of this interaction as well as the cause for the difference between the *in vivo* and *in vitro* results have never been explored.

The availability of phosphorylation-site specific antibodies to phospholamban, which precisely discriminate between Ser^{16} and Thr^{17} phosphorylation sites (12), prompted us to reexamine the issue. Combination of this technique with the quantitative assessment of phospholamban phosphorylation by radiochemical labeling of ATP pools and simultaneous measurements of mechanical parameters allowed us to characterize the PKA and CaMKII-dependent mechanisms of phospholamban phosphorylation in the intact heart and their relative physiological roles on cardiac performance.

EXPERIMENTAL PROCEDURES

Heart Perfusions—Experiments were performed in isolated hearts from male Wistar rats (250–350 g body wt) perfused according to the Langendorff technique as described previously (8). 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. 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 either sewing an isometric strain gauge arch (Micro Measurements, type MA-06–030LB-120) to the left ventricular wall or passing into the left ventricle a latex balloon connected to a pressure transducer (Namic, perceptor DT disposable transducer). The initial length of the gauge was set by stretching the segment attached by approximately 30%. The balloon was filled with aqueous solution to achieve a left ventricular

* This work was supported in part by a grant from Fundación Antorchas and the British Council. 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.

‡ Established Investigators of Consejo Nacional de Investigaciones Científicas y Técnicas (Argentina).

§ Fellow from Comisión de Investigaciones Científicas de la Provincia de Buenos Aires (Argentina).

¶ To whom correspondence should be addressed. Tel.: 54–21-34833; Fax: 54–21-25–5861; E-mail: cicme@isis.unlp.edu.ar.

[1] The abbreviations used are: SR, sarcoplasmic reticulum; Ca^{2+}-ATPase, ; PKA, cAMP-dependent protein kinase; CaMKII, Ca^{2+}-calmodulin-dependent protein kinase; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; PP1, phosphatase type 1; OA, okadaic acid; PSS, physiological salt solution; PSP, poststimulation potentiation.

end-diastolic pressure of 8–14 mm Hg. No differences were observed between the mechanical data obtained by measuring either the isometric tension or the isovolumic pressure, and they were considered together for statistical analysis. Hearts were perfused with PSS for 10–15 min for stabilization and then for the next 4 min with either PSS (control) or different interventions, as described under "Results." To quantify ^{32}P incorporation into phospholamban, hearts were perfused for 60 min with recirculation with PSS containing 10 μCi/ml $^{32}P_i$ after the stabilization period and previously to the interventions assessed. 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—Membrane vesicles were prepared as described previously (8), except that the pulverized tissue from each heart was homogenized in 6 volumes of a medium containing (in mM): 30 KH_2PO_4 (pH 7.0), 5 Na_2EDTA, 25 NaF, 300 sucrose, 1 phenylmethylsulfonyl fluoride, and 1 benzamidine. Samples from ^{32}P-labeled perfused hearts were homogenized in the same medium except that the phosphate was replaced by 20 mM Tris-HCl (pH 7.0). Protein was measured by the method of Bradford (13) using bovine serum albumin as standard. The yield was 1–2 mg of membrane vesicles protein per g of cardiac tissue.

Electrophoresis and Western Blot Analysis—SDS-PAGE was performed using 10% acrylamide slab gels according to Porzio and Pearson (14) as described previously (8). Samples for electrophoresis were not boiled unless stated. For immunological detection of phospholamban phosphorylation sites, 10 μg of membrane protein were electrophoresed per gel lane. Proteins were transferred to PVDF membranes (Immobilon-P, Millipore) and probed according to Drago and Colyer (12) with monoclonal antibody to phospholamban (1:5000) or polyclonal antibodies raised to a phospholamban peptide (residues 9–19) phosphorylated at Ser^{16} (1:10,000) or at Thr^{17} (1:5000) (PhosphoProtein Research, UK). Immunoreactivity was visualized by peroxidase-conjugated antibodies using a peroxidase-based chemiluminescence detection kit (Boehringer Mannheim, Germany). The signal intensity of the bands on the film was quantified by optical densitometric analysis. To assess ^{32}P incorporation into phospholamban, 300 μg of membrane protein were electrophoresed per gel lane. Gels were run in duplicate in order to use one of them for autoradiography and the other for liquid-scintillation counting. Quantitative results were expressed as pmol of ^{32}P incorporated into phospholamban per mg of SR protein based on the specific activity of ^{32}P in phosphocreatine (15).

Phosphorylation of Isolated SR Membranes—Phosphorylation of SR membrane vesicles was carried out at 30 °C in 0.1 ml of reaction medium containing 50 μg SR protein, 50 mM Tris-HCl (pH 7.0), 5 mM EGTA, 5 mM Mg-acetate, and 200 μM ATP. PKA-dependent phosphorylation was catalyzed by 30 units/ml of the PKA-catalytic subunit. Alternatively, PKA-catalytic subunit and EGTA were omitted, and phosphorylation was carried out by the endogenous CaMKII present in the SR membranes in the presence of 0.5 mM Cl_2Ca, 1 μM calmodulin, and 1 μM protein kinase A inhibitor peptide 5–24 amide. Reaction was stopped after 1 min (PKA) or 5 min (CaMKII) with SDS sample buffer.

Other Assays—Myocardial cAMP content was measured as described previously (8) by radioimmunoassay (16) using a commercially available kit (DuPont NEN) with acetylation of the samples. Results are expressed as pmol of cAMP per mg of wet weight. PP1 activity was assayed by measuring dephosphorylation of ^{32}P-labeled phosphorylase *a* (17). One activity unit was that amount of enzyme that catalyzes the release of 1 μmol of ^{32}P/min.

Statistics—All data are expressed as the mean ± S.E. of *n* preparations. Student's *t* test for paired or unpaired observations (mechanical and biochemical results, respectively) was used to test for statistical differences. $p < 0.05$ was considered statistically significant.

RESULTS

Immunodetection of Site-specific Phosphorylated Phospholamban—Specific antibodies to phospholamban, Ser^{16} and Thr^{17}-phosphorylated phospholamban peptides, were tested in SR membrane vesicles phosphorylated by the catalytic subunit of PKA or by the endogenous SR CaMKII (Fig. 1). The monoclonal antibody to phospholamban identified both the pentameric form of phospholamban (Fig. 1*A*, *lanes a* and *c*) and the monomeric form obtained by boiling the sample prior to electrophoresis (Fig. 1*A*, *lanes b* and *d*). Antibody to Ser^{16} phosphopeptide only recognized phospholamban phosphorylated by PKA (Fig. 1*B*, *lanes a* and *b*) and ignored CaMKII-phosphorylated phospholamban (Fig. 1*B*, *lanes c* and *d*). Conversely, antibody to Thr^{17} phosphopeptide exclusively recognized CaMKII-phosphorylated phospholamban (Fig. 1*C*, *lanes c* and *d*). Antibodies to Ser^{16} phosphopeptide and to Thr^{17} phosphopeptide did not recognize unphosphorylated phospholamban (data not shown).

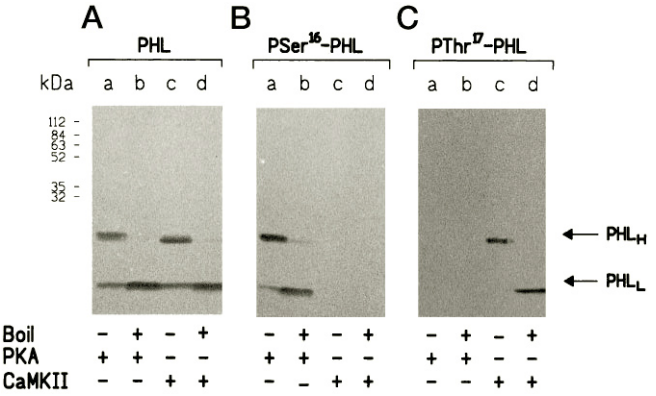

FIG. 1. **Immunodetection of site-specific phosphorylated phospholamban.** Cardiac SR membrane vesicles were phosphorylated with 200 μM ATP at 30 °C by either 30 units/ml of the catalytic subunit of cAMP-dependent protein kinase (*PKA*, *lanes a* and *b*) or in the presence of 0.5 mM Cl_2Ca and 1 μM calmodulin to stimulate the intrinsic Ca^{2+}-calmodulin-dependent protein kinase activity (*CaMKII*, *lanes c* and *d*). Reaction was stopped either after 1 min (PKA) or 5 min (CaMKII) with SDS sample buffer. Samples were solubilized at room temperature (*lanes a* and *c*) or at 100 °C for 5 min (*lanes b* and *d*). 10 μg of protein were loaded onto each lane of SDS-PAGE gels. Proteins were transferred to PVDF membranes. Blots were probed with monoclonal antibody to phospholamban (1:5000, *panel A, PHL*), or polyclonal antibodies to Ser^{16} phosphopeptide (1:10000, *panel B, PSer^{16}-PHL*) and Thr^{17} phosphopeptide (1:5000, *panel C, PThr^{17}-PHL*). Antibody binding was visualized using a chemiluminescence detection kit. PHL_H and PHL_L designate the pentameric and monomeric forms of phospholamban, respectively.

Isoproterenol Concentration-dependent Induced Increase in Phospholamban Phosphorylation. Independence of the PKA Cascade—Fig. 2, *panel A*, shows an autoradiograph of SR membrane vesicles isolated from hearts perfused with ^{32}P in control conditions and in the presence of 3, 30, and 300 nM isoproterenol. Isoproterenol increased phosphorylation of phospholamban and reached a "plateau" at 30 nM. *Panel B* shows overall results of this experimental series.

Fig. 3*A*, *upper panel*, shows immunoblots of SR membrane vesicles obtained from hearts perfused with increasing isoproterenol concentrations under conditions of normal calcium supply to the myocardium (1.35 mM $[Ca]_o$). β-adrenergic stimulation induced phosphorylation of both Ser^{16} and Thr^{17} residues of phospholamban. Mean values of Ser^{16} and Thr^{17} phospholamban phosphorylation obtained from optical densitometric analysis of three different experiments of this type are shown by the open symbols in Fig. 3*B*. The results indicate that the isoproterenol concentration-dependent increase in phospholamban phosphorylation (Fig. 2) is due to an isoproterenol concentration-dependent increase in the phosphorylation of both Ser^{16} and Thr^{17} residues. In all cases, a maximum was reached at 30 nM isoproterenol. Table I shows mean values of intracellular cAMP levels and of mechanical parameters obtained at the different isoproterenol concentrations.

In an attempt to diminish Ca^{2+} supply to the myocardium and therefore block phospholamban phosphorylation by CaMKII, hearts were perfused with the same isoproterenol concentrations as above but in the presence of 0.07 mM $[Ca]_o$ plus 400 nM nifedipine. Myocardial contractility was abolished at any of the isoproterenol concentrations studied. Under these conditions, an isoproterenol concentration-dependent phosphorylation of Ser^{16} was observed without detectable changes in

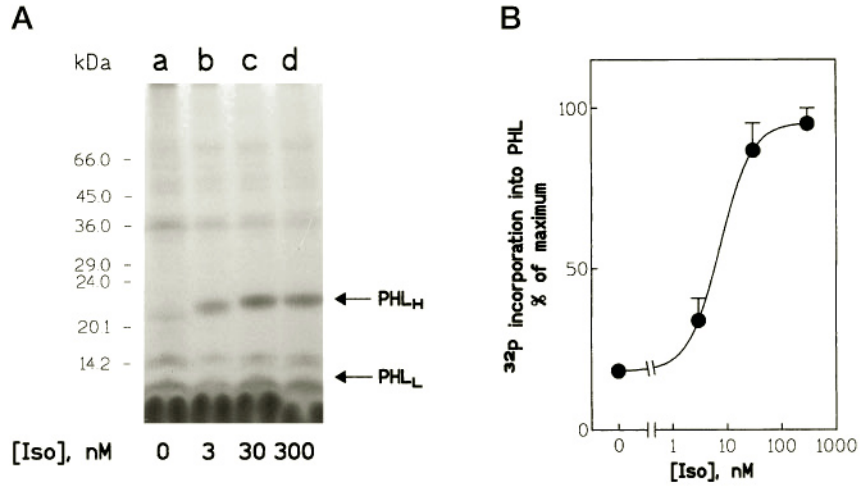

FIG. 2. **Isoproterenol concentration-dependent increase in ^{32}P incorporation into phospholamban.** *Panel A*, autoradiograph of SR membrane vesicles isolated from rat hearts perfused with ^{32}P and then without (*lane a*) or with different isoproterenol concentrations (*Iso, lanes b* to *d*). Samples (300 μg) were subjected to SDS-PAGE and autoradiography. PHL_H and PHL_L designate the pentameric and monomeric forms of phospholamban, respectively. *Panel B*, isoproterenol dose-response curve for ^{32}P incorporation into phospholamban. Results are expressed as percentage of the maximal ^{32}P incorporation into phospholamban achieved in each experimental series (n = 4). Maximal ^{32}P incorporation into phospholamban was 278.0 ± 26.1 pmol/mg of SR protein.

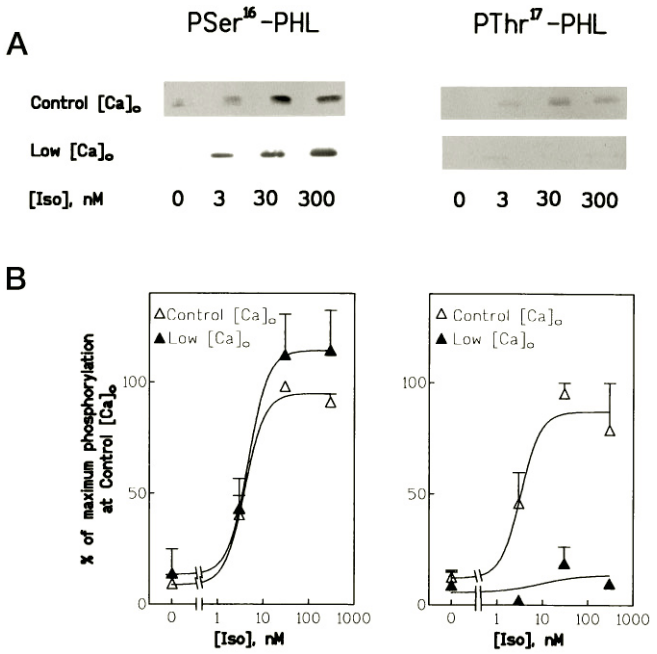

FIG. 3. **Isoproterenol concentration-dependent increase in phosphorylation of Ser^{16} and Thr^{17} residues of phospholamban.** *Panel A*, immunoblots of SR membrane vesicles isolated from hearts perfused with different isoproterenol concentrations (*Iso*) at 1.35 mM $[Ca]_o$ (*Control* $[Ca]_o$) or at 0.07 mM $[Ca]_o$ + 400 nM nifedipine (*Low* $[Ca]_o$). 10 μg of SR protein were resolved by SDS-PAGE and transferred to PVDF membranes. Blots were probed with anti-Ser^{16} phosphopeptide (*PSer^{16}-PHL*) and anti-Thr^{17} phosphopeptide (*PThr^{17}-PHL*). Antibody binding was visualized using a chemiluminescence detection kit. *Panel B*, mean ± S.E. values obtained after the densitometric analysis of the signal of three immunoblots. Results are expressed as percentage of the maximal signal achieved in each experimental series. At low $[Ca]_o$, 30 nM isoproterenol at 1.35 mM $[Ca]_o$, run in parallel, was regarded as 100%.

phosphorylation of Thr^{17} (Fig. 3*A*, *lower panel*). Mean results of three different experiments of this type are shown by the filled symbols in Fig. 3*B*. At each isoproterenol concentration, Ser^{16} was phosphorylated to the same extent, independently of the degree of CaMKII-induced phosphorylation of Thr^{17}. These findings indicate that PKA-dependent phospholamban phosphorylation is independent of the CaMKII pathway in the intact functioning heart.

Additivity of PKA- and CaMKII-dependent Phosphorylation of Phospholamban in the Intact Heart—To study the relative contribution of both phosphorylation sites to the total phospholamban phosphorylation after β-adrenoreceptor stimulation, hearts were perfused with ^{32}P, and phosphorylation of phospholamban was quantified at the "plateau" of the dose-response curve to isoproterenol (30 nM), under conditions of normal $[Ca]_o$ (1.35 mM) and diminished Ca^{2+} supply to the myocardium (0.25 mM $[Ca]_o$ and 0.07 mM $[Ca]_o$ plus 400 nM nifedipine). The decrease in Ca^{2+} supply gradually decreased phospholamban phosphorylation (Fig. 4*A*) and myocardial relaxation without changes in cAMP levels (Table I). Immunodetection of the site-specific phosphorylated phospholamban revealed that the decrease in phospholamban phosphorylation is exclusively due to a decrease in Thr^{17} phosphorylation (Fig. 4*B*). Note that the isoproterenol-induced increase in Ser^{16} phosphorylation was the same at any of the $[Ca^{2+}]_o$ assayed. Under conditions of extremely low Ca^{2+} supply to the myocardium, in which Thr^{17} phosphorylation was virtually absent, total phospholamban phosphorylation decreased by approximately 50%. These findings demonstrate the additivity of PKA and CaMKII pathways of phospholamban phosphorylation, in agreement with the *in vitro* results (4). Furthermore, both mechanisms contribute to the relaxant action of β-adrenergic stimulation (Table I).

CaMKII-dependent Phospholamban Phosphorylation. Role of Phosphatases—To address the participation of phosphatases in the degree of phospholamban phosphorylation, rat hearts were perfused with ^{32}P at two different $[Ca]_o$ in the absence and in the presence of the phosphatase inhibitor OA (0.1 μM). This OA concentration decreased PP1 activity by 82.5 ± 1.5%. Only in the presence of OA did the increase in $[Ca]_o$ enhance phospholamban phosphorylation (Fig. 5). This enhanced phosphorylation of phospholamban was associated with a significant increase in contractility and a decrease in half relaxation time (Table I). In 4 out of 11 experiments, the addition of OA at 3.85 mM $[Ca]_o$ produced a heart contracture. The cause for this contracture was not explored in the present experiments but might be due to the effects of OA on phosphatases other than those regulating phospholamban phosphorylation (18, 19). The failure to detect phospholamban phosphorylation after increasing $[Ca]_o$, in the absence of OA, was also observed when contractility was increased by poststimulation potentiation (PSP) (9) to levels similar to those evoked by maximal β-adrenergic stimulation (PSP of 88 ± 9% *versus* isoproterenol of 72 ± 7%). Phospholamban phosphorylation in pmol of ^{32}P/mg of SR protein was: PSP, 20.0 ± 1.0 and control, 24.7 ± 1.2. Immunological detection of the two phosphorylation sites of phospholamban (Fig. 6*A*) showed that the increase in $[Ca]_o$ did not increase phosphorylation of either Thr^{17} or Ser^{16} residues. The same increase in $[Ca]_o$ in the presence of 1 μM OA increased phosphorylation of Thr^{17} without affecting Ser^{16} (Fig. 6*A*). This

TABLE I

Effects of different interventions on mechanical parameters and intracellular cAMP levels in the intact heart

Rat hearts were perfused with the indicated interventions and then freeze-clamped and processed as described under "Experimental Procedures." Results are mean ± SEM of *n* hearts (parentheses). PSS: physiological salt solution; Nife: nifedipine; ND: not determined. Δ, indicate differences with respect to control. Control for mechanical data are values obtained immediately before the intervention. Control for biochemical data are values obtained in the PSS group.

Treatment	Maximal rate of contraction	Half relaxation time	cAMP
	% of control	Δ *msec*	*pmol/mg wet wt*
PSS			
1.35 mM $[Ca]_o$	99.97 ± 1.72 (25)	−0.09 ± 0.73 (24)	0.823 ± 0.099 (6)
Isoproterenol			
3 nM			
1.35 mM $[Ca]_o$	135.21 ± 7.02[a] (10)	−10.75 ± 0.90[a] (10)	1.097 ± 0.036[a] (3)
30 nM			
1.35 mM $[Ca]_o$	172.42 ± 7.01[a] (9)	−19.95 ± 1.78[a] (19)	1.696 ± 0.054[a] (6)
300 nM			
1.35 mM $[Ca]_o$	187.52 ± 21.17[a] (10)	−19.11 ± 0.56[a] (10)	1.759 ± 0.204[a] (3)
30 nM			
0.25 mM $[Ca]_o$	106.05 ± 8.80 (9)	−9.67 ± 1.69[a] (9)	1.692 ± 0.096[a] (3)
30 nM			
0.07 mM $[Ca]_o$ + 400 nM Nife	ND	ND	1.610 ± 0.178[a] (3)
Calcium			
3.85 mM $[Ca]_o$	135.57 ± 8.91[a] (13)	−1.51 ± 2.30 (12)	0.715 ± 0.082 (3)
Okadaic acid			
0.1 μM			
1.35 mM $[Ca]_o$	104.95 ± 5.33 (9)	−0.80 ± 2.86 (7)	ND
0.1 μM			
3.85 mM $[Ca]_o$	160.47 ± 10.18[a] (7)	−8.57 ± 3.87 (7)	0.759 ± 0.142 (3)

[a] $p < 0.05$ when compared to control.

concentration of OA decreased PP1 activity by 97.9 ± 1.0%. The overall results of this series are shown in Fig. 6*B*. These findings indicate that in the intact functioning heart CaMKII-dependent phospholamban phosphorylation can be detected in the absence of intracellular cAMP increases, provided phosphatases are inhibited.

DISCUSSION

Phosphorylation site-specific antibodies are a novel experimental tool to recognize a phosphorylated epitope of a protein. The phosphorylation site-specific antibodies to phospholamban (12) used in the present experiments failed to detect the unphosphorylated protein and proved to be highly specific in the discrimination between the two sites of phosphorylation of phospholamban since no cross-reactivity with the other site of phosphorylation was observed (Fig. 1). The combination of this technique with the classical isotopic labeling technique of quantification of phospholamban phosphorylation, along with simultaneous measurements of mechanical parameters, allowed us a detailed characterization of the two signaling cascades of phospholamban phosphorylation, the relationships between them, and their physiological significance in the intact heart.

In agreement with the *in vitro* findings, the present results demonstrate that in the intact functioning heart, PKA- and CaMKII-dependent pathways of phospholamban phosphorylation may work independently of each other (Figs. 3 and 6) and that when both mechanisms are operating they have an additive action (Fig. 4). Previous works have consistently shown that CaMKII-dependent phospholamban phosphorylation and changes in myocardial relaxation can only occur in the intact heart when cAMP levels within the cell increase (7–11). This finding was interpreted as an interrelationship between PKA and CaMKII cascades, which would favor dual site phosphorylation of phospholamban after β-adrenoreceptor stimulation. This conclusion was in sharp contrast with the independence of both pathways described in the *in vitro* systems. The reason for the apparent discrepancy between the *in vitro* and the *in vivo* results as well as the nature of the interaction between the two phosphorylation cascades in the intact heart are not yet understood. Several mechanisms have been considered to explain this interaction, among which are the following. 1) Only the increases in intracellular Ca^{2+} evoked by cAMP-dependent mechanisms are large enough to activate CaMKII pathway. 2) CaMKII cascade could be activated only by compartmentalized increases in Ca^{2+} evoked by cAMP increases. 3) CaMKII could be activated by cAMP-dependent mechanisms unrelated to intracellular Ca^{2+} increase. 4) Phosphatases that dephosphorylate phospholamban could be inhibited by a PKA-dependent mechanism. The fact that increases in contractility (reflecting cytosolic Ca^{2+}) similar to that evoked by isoproterenol, *i.e.* PSP, failed to phosphorylate phospholamban allows to rule out the first possibility. Similar conclusions were previously obtained (9). Furthermore, and relevant to the first three possibilities, we are presenting evidence showing that activation of CaMKII cascade and phosphorylation of Thr^{17} residue could be detected even in the absence of high cAMP levels (Fig. 6). The present findings give support to the fourth hypothesis. Our results indicate that the nature of the interaction between PKA and CaMKII cascades of phospholamban phosphorylation lies in a basic mechanism underlying any phosphorylation process, *i.e.* the degree of phosphatase activity in the different experimental conditions. The major phosphatase that dephosphorylates phospholamban is a form of PP1 associated to the SR (20). Phosphorylation of Thr^{17} residue in the intact heart could be

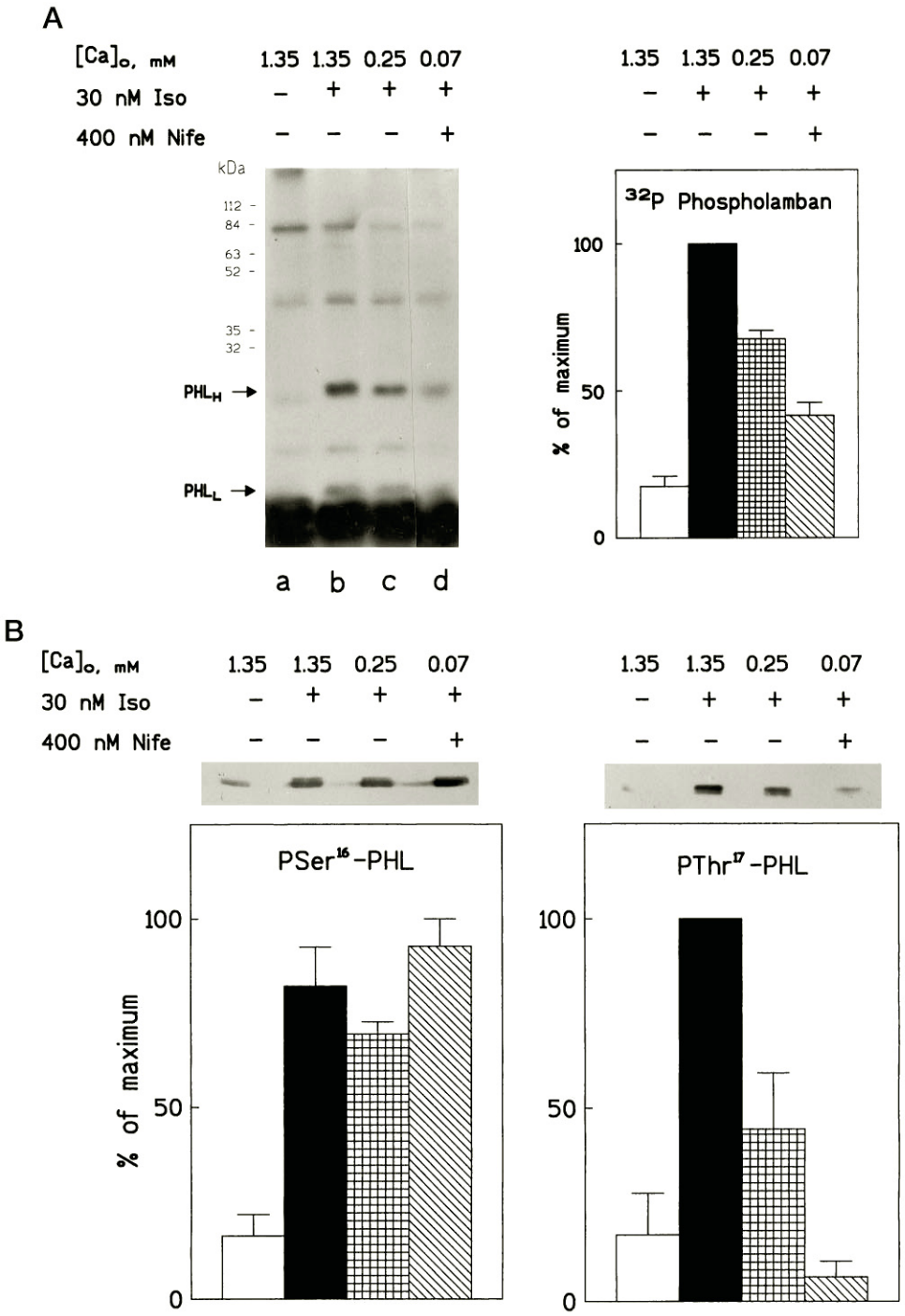

FIG. 4. **Effects of decreasing Ca^{2+} supply to the myocardium on isoproterenol-induced phospholamban phosphorylation.** *Panel A, left*, autoradiograph of SR membranes isolated from rat hearts perfused with ^{32}P in the absence (*lane a*) or the presence (*lanes b* to *d*) of 30 nM isoproterenol (*Iso*) simultaneously given with interventions that gradually decrease the Ca^{2+} supply to the myocardium, low $[Ca]_o$ with or without nifedipine (*Nife*). PHL_H and PHL_L designate the pentameric and monomeric forms of phospholamban, respectively. *Panel A, right*, overall results ($n = 3$) of ^{32}P incorporation into phospholamban obtained under the experimental conditions depicted by the autoradiograph. Results are expressed as percentage of the maximal ^{32}P incorporation into phospholamban achieved in each experimental series. Maximal ^{32}P incorporation into phospholamban was 184.0 ± 8.0 pmol/mg of SR protein. *Panel B*, blots and overall results of the immunodetection of the site-specific phosphorylated phospholamban (*PSer*^{16}*-PHL* and *PThr*^{17}*-PHL*) after the same treatment. Mean ± S.E. values of the densitometric analysis of the signal of three immunoblots are shown. Results are expressed as percentage of the maximal signal achieved in each experimental series.

detected in two situations, at high intracellular cAMP levels and in the presence of OA. It has been proposed that SR-associated PP1 could be inhibited by PKA-dependent phosphorylation by two different but related mechanisms. First, as described for PP1 associated to glycogen particles (21) and to SR in skeletal muscle (22, 23), PKA phosphorylation of the PP1-regulatory subunit would release the catalytic (C)-subunit, which would prevent PP1 from dephosphorylating phospholamban (20). Second, the thermostable protein inhibitor 1, when phosphorylated by PKA, becomes a potent inhibitor of PP1 C-subunit (24). Evidence for an isoproterenol-induced phosphorylation and increased activity of inhibitor 1 and for a reduction in SR-associated PP1 activity have been reported in the intact heart and isolated myocytes (25–27). PP1 is also

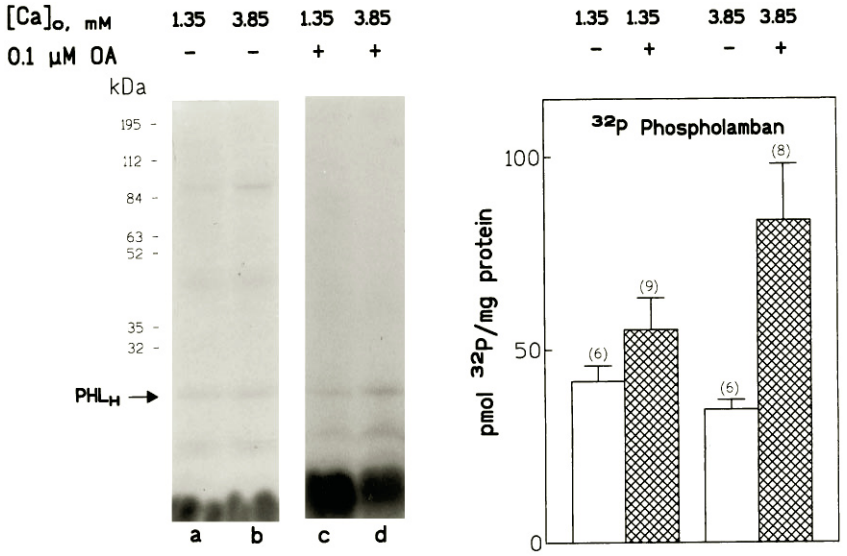

FIG. 5. **Increasing $[Ca]_o$ in the presence of okadaic acid enhanced phospholamban phosphorylation.** *Left*, SR membranes were isolated from ^{32}P-perfused rat hearts at two different $[Ca]_o$ in the absence (*lanes a* and *b*) or the presence (*lanes c* and *d*) of 0.1 μM okadaic acid (*OA*). Samples (300 μg) were subjected to SDS-PAGE and autoradiography. PHL_H designates the pentameric form of phospholamban. *Right*, bars show overall results of ^{32}P incorporation into phospholamban. The number of experiments in each treatment group is indicated in parentheses.

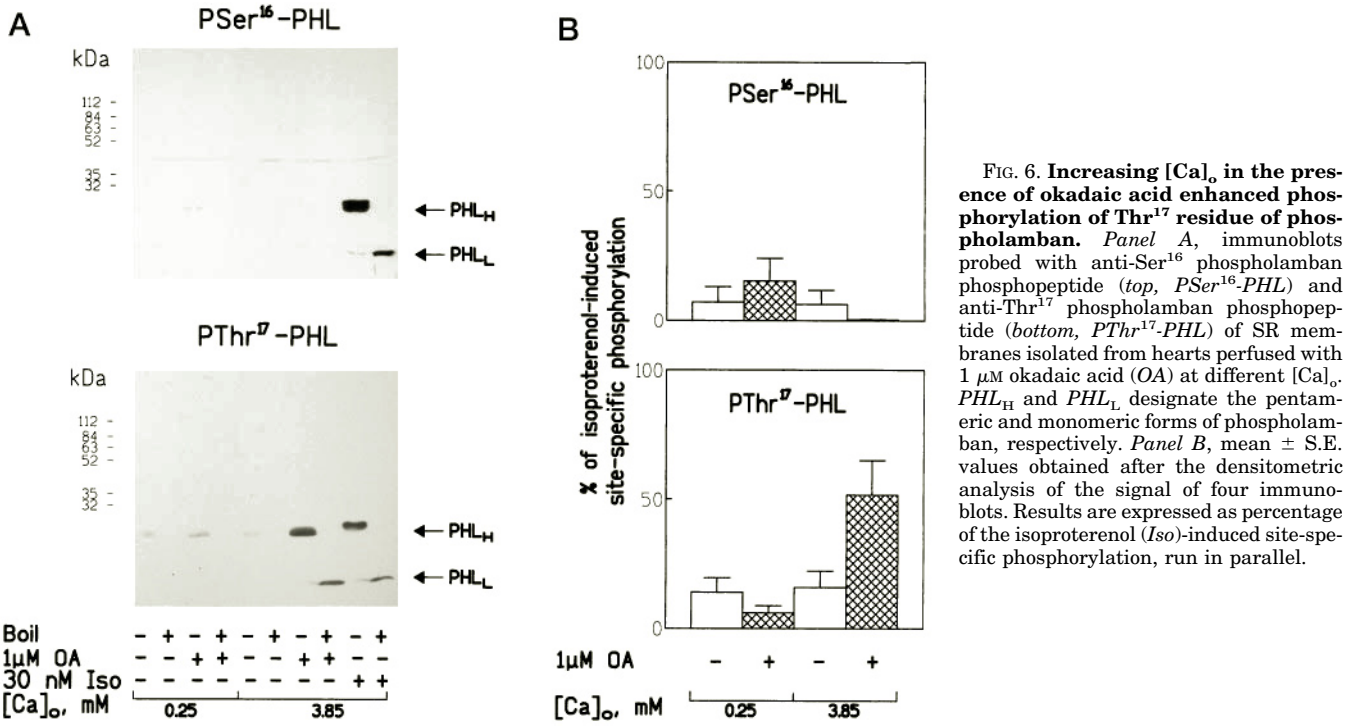

FIG. 6. **Increasing $[Ca]_o$ in the presence of okadaic acid enhanced phosphorylation of Thr^{17} residue of phospholamban.** *Panel A*, immunoblots probed with anti-Ser^{16} phospholamban phosphopeptide (*top, PSer*^{16}*-PHL*) and anti-Thr^{17} phospholamban phosphopeptide (*bottom, PThr*^{17}*-PHL*) of SR membranes isolated from hearts perfused with 1 μM okadaic acid (*OA*) at different $[Ca]_o$. PHL_H and PHL_L designate the pentameric and monomeric forms of phospholamban, respectively. *Panel B*, mean ± S.E. values obtained after the densitometric analysis of the signal of four immunoblots. Results are expressed as percentage of the isoproterenol (*Iso*)-induced site-specific phosphorylation, run in parallel.

inhibited by OA (18). We have found that increases in $[Ca]_o$, in the presence of OA, phosphorylated phospholamban exclusively in Thr^{17}. This phosphorylation was associated to a decrease in half relaxation time. Higher OA concentrations were described to produce an increase in phospholamban phosphorylation and an enhanced myocardial relaxation (28). A faster intracellular Ca^{2+} decline induced by OA has also been observed in isolated myocytes (29). Unfortunately, none of these studies looked for sites of phosphorylation of phospholamban. In the present results, both situations in which we were able to detect Thr^{17} phosphorylation, high cAMP and OA, have a common feature, and this is the inhibition of phosphatases.

In contrast to the *in vivo* results, phosphatase inhibition was not required to detect phosphorylation of either Ser^{16} or Thr^{17} residues of phospholamban in the *in vitro* systems (Fig. 1). This might be due to the already low phosphatase activity of the SR membrane preparation commonly used for the *in vitro* assays. It has been shown that PP1 activity decreased during the standard procedure of isolation of SR membrane vesicles (20) and that high salt treatment (used to wash out the membranes from myofibrillar proteins) also inhibits SR-associated PP1 activity (22).

For several years, different laboratories including our own, attempted to define the physiological role of the CaMKII pathway of phosphorylation of phospholamban (6–11, 29). We are presenting evidence that indicate that both phosphorylation of phospholamban as well as the physiological expression of this phosphorylation, *i.e.* the increase in myocardial relaxation and the consequent increase in contractility, requires simultaneous stimulation of protein kinases and inhibition of phosphatases. Physiologically, this requisite is only filled by β-adrenergic stimulation. Although in the absence of high intracellular cAMP levels, the physiological meaning of CaMKII cascade appears as negligible, in its presence it is not. The present results indicate that under maximal β-adrenergic stimulation, activation of CaMKII cascade accounts for about 50% of phos-

pholamban phosphorylation. This CaMKII-induced phospholamban phosphorylation is closely associated with an increase in the relaxant capacity of the intact ventricle.

Acknowledgments—We thank Professor Philip Cohen, University of Dundee, UK, for advice on PP1 assay and the very kind gift of purified PP1.

REFERENCES

1. Tada, M., Yamada, M., Kadoma, M., Inui, M., and Ohmori, F. (1982) *Mol. Cell. Biochem.* **46,** 73–95
2. Suzuki, T., and Wang, J. H. (1986) *J. Biol. Chem.* **261,** 7018–7023
3. Simmerman, H. K. B., Collins, J. H., Theibert, J. L., Wegener, A. D., and Jones, L. R. (1986) *J. Biol. Chem.* **261,** 13333–13341
4. Bilezikjian, L. M., Kranias, E. G., Potter, J. D., and Schwartz, A. (1981) *Circ. Res.* **49,** 1356–1362
5. Wegener, A. D., Simmerman, H. K. B., Lindenmann, J. P., and Jones, L. R. (1989) *J. Biol. Chem.* **264,** 11468–11474
6. Lindemann, J. P., Jones, L. R., Hathaway, D. R., Henry, B. G., and Watanabe, A. M. (1983) *J. Biol. Chem.* **258,** 464–471
7. Lindemann, J. P., and Watanabe, A. M. (1985) *J. Biol. Chem.* **260,** 4516–4525
8. Vittone, L., Mundiña, C., Chiappe de Cingolani, G., and Mattiazzi, A. (1990) *Am. J. Physiol.* **258,** H318–H325
9. Napolitano, R., Vittone, L., Mundiña, C., Chiappe de Cingolani, G., and Mattiazzi, A. (1992) *J. Mol. Cell. Cardiol.* **24,** 387–396
10. Vittone, L., Mundiña, C., Chiappe de Cingolani, G., and Mattiazzi, A. (1993) *Mol. Cell. Biochem.* **124,** 33–42
11. Mattiazzi, A., Hove-Madsen, L., and Bers, D. M. (1994) *Am. J. Physiol.* **267,** H812–H820
12. Drago, G., and Colyer, J. (1994) *J. Biol. Chem.* **269,** 25073–25077
13. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254
14. Porzio, M. A., and Pearson, A. M. (1977) *Biochim. Biophys. Acta* **490,** 27–34
15. Barron, J. T., Bárány, M., and Bárány, K. (1979) *J. Biol. Chem.* **254,** 4954–4956
16. Steiner, A. L., Parker, C. W., and Kipnis, D. M. (1984) *J. Biol. Chem.* **247,** 1106–1113
17. Cohen, P., Alemany, S., Hemmings, B. A., Resink, T., Stralfors, P., and Lim Tung, H. Y. (1988) *Methods Enzymol.* **159,** 390–408
18. Haystead, T. A. J., Sim, A. T. R., Carling, D., Honnor, R. C., Tsukitani, Y., Cohen, P., and Hardie, D. G. (1989) *Nature* **337,** 78–81
19. Hescheler, J., Mieskes, G., Ruegg, J. C., Takai, A., and Trautwein, W. (1988) *Pfluegers Arch. Eur. J. Physiol.* **412,** 248–252
20. MacDougall, L. K., Jones, L. R., and Cohen, P. (1991) *Eur. J. Biochem.* **196,** 725–734
21. Hubbard, M. J., and Cohen, P. (1989) *Eur. J. Biochem.* **186,** 711–716
22. Hubbard, M. J., Dent, P., Smythe, C., and Cohen, P. (1990) *Eur. J. Biochem.* **189,** 243–249
23. Vittone, L., Mundiña, C., and Cohen, P. (1993) *Medicina, Buenos Aires* **53,** Suppl. II, 61
24. Cohen, P. (1989) *Annu. Rev. Biochem.* **58,** 453–508
25. Ahmad, Z., Green, F. J., Subuhi, H. S., and Watanabe, A. M. (1989) *J. Biol. Chem.* **264,** 3859–3863
26. Neumann, J., Gupta, R. C., Schmitz, W., Scholz, H., Nairn, A. C., and Watanabe, A. M. (1991) *Circ. Res.* **69,** 1450–1457
27. Gupta, R. C., Neumann, J., Watanabe, A. M., Lesch, M., and Sabbah, H. N. (1996) *Am. J. Physiol.* **270,** H1159–H1164
28. Neumann, J., Boknik, P., Herzig, S., Schmitz, W., Scholz, H., Gupta, R. C., and Watanabe, A. M. (1993) *Am. J. Physiol.* **265,** H257–H266
29. Bassani, R. A., Mattiazzi, A., and Bers, D. M. (1995) *Am. J. Physiol.* **268,** H703–H712