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

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Cecilia Mundiña-Weilenmann‡, Leticia Vittone‡, Manuel Ortales§, Gladys Chiappe de Cingolani‡, and Alicia Mattiazzip¶
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[^32P] 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,[^32P] 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[^2]PO[^4], 1.1 MgCl[^2], 11.1 glucose, and 0.04 Na[^2]EDTA. 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...
end-diastolic pressure of 8–14 mm Hg. No differences were observed between the 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, 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): 10 EGTA, 5 NaF, 300 sucrose, 10 hemethylsulfonyl 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 to Ser16 (1:10,000) or at Thr 17 (1:5000) (PhosphoProtein Research, UK). Immunoreactivity was visualized by peroxidase-conjugated antibodies directed to Ser16 phosphopeptide (1:10000, body to phospholamban (1:5000, visualized using a chemiluminescence detection kit. Antibody to Thr17 phosphopeptide exclusively recognized CaMKII-phosphorylated phospholamban (Fig. 1C, lanes c and d). Antibodies to Ser16 phosphopeptide and to Thr17 phosphopeptide did not recognize unphosphorylated phospholamban (data not shown).

**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.

**RESULTS**

**Immunodetection of Site-specific Phosphorylated phospholamban**—Specific antibodies to phospholamban, Ser16 and Thr17-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. 1A, lanes a and c) and the monomeric form obtained by boiling the sample prior to electrophoresis (Fig. 1A, lanes b and d). Antibody to Ser16 phosphopeptide only recognize phosphorylated phospholamban phosphorylated by PKA (Fig. 1B, lanes a and b) and ignored CaMKII-phosphorylated phospholamban (Fig. 1B, lanes c and d). Conversely, antibody to Thr17 phosphopeptide exclusively recognizes CaMKII-phosphorylated phospholamban (Fig. 1C, lanes c and d).

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

**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 CaCl2 and 1 μM calmodulin to stimulate the intrinsic Ca2+-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, phospho-PR, PHL), or polyclonal antibodies to Ser16 phosphopeptide (1:10000, panel B, P Ser16- PPHL) and Thr17 phosphopeptide (1:5000, panel C, P Thr17-PHL). Antibody binding was visualized using a chemiluminescence detection kit. PHLm and PHLc designate the pentameric and monomeric forms of phospholamban, respectively.

**Fig. 1. Immunodetection of site-specific phosphorylated phospholamban.**

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**Table I**

| Assay                        | Conditions | Values (pmol $^{32}$P incorporated per mg of SR protein) |
|------------------------------|------------|---------------------------------------------------------|
| PKA                          | 10 nM isoproterenol | 55 ± 2 (control) 102 ± 3 (isoproterenol) |
| CaMKII                       | 30 nM isoproterenol | 70 ± 3 (control) 120 ± 5 (isoproterenol) |

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**Fig. 2.** Immunoblot analysis of SR membrane vesicles phosphorylated by PKA or CaMKII. A. Lane a, control; lane b, PKA; lane c, CaMKII. B. Lane a, 5 nM isoproterenol; lane b, 30 nM isoproterenol; lane c, 300 nM isoproterenol. C. Lane a, control; lane b, PKA; lane c, CaMKII.

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**Fig. 3.** A. Upper panel, shows immunoblots of SR membrane vesicles obtained from hearts perfused with $^{32}$P in control conditions and in the presence of 3, 30, and 300 nM isoproterenol. B. Lower panel, shows quantification of PKA and CaMKII phosphorylation. C. Lower panel, shows quantification of PKA and CaMKII phosphorylation.
Additivity of PKA- and CaMKII-dependent Phosphorylation

FIG. 3. Isoproterenol concentration-dependent increase in phosphorylation of Ser\textsuperscript{16} and Thr\textsuperscript{17} residues of phospholamban. Panel A, immunoblots of SR membrane vesicles isolated from hearts perfused with 32P at two different [Ca\textsubscript{o}] in the absence and in the presence of OA (0.1 μM). Only in the presence of OA did the increase in [Ca\textsubscript{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\textsubscript{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\textsubscript{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 32P/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. 6A) showed that the increase in [Ca\textsubscript{o}] did not increase phosphorylation of either Thr\textsuperscript{17} or Ser\textsuperscript{16} residues. The same increase in [Ca\textsubscript{o}] in the presence of 1 μM OA increased phosphorylation of Thr\textsuperscript{17} without affecting Ser\textsuperscript{16} (Fig. 6A). This

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phosphorylation of Thr\textsuperscript{17} (Fig. 3A, lower panel). Mean results of three different experiments of this type are shown by the filled symbols in Fig. 3B. At each isoproterenol concentration, Ser\textsuperscript{16} was phosphorylated to the same extent, independently of the degree of CaMKII-induced phosphorylation of Thr\textsuperscript{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-adrenergic receptor stimulation, hearts were perfused with 32P, and phosphorylation of phospholamban was quantified at the “plateau” of the dose-response curve to isoproterenol (30 nM), under conditions of normal [Ca\textsubscript{o}] (1.35 mM) and diminished Ca\textsuperscript{2+} supply to the myocardium (0.25 mM [Ca\textsubscript{o}] and 0.07 mM [Ca\textsubscript{o}] plus 400 nM nifedipine). The decrease in Ca\textsuperscript{2+} supply gradually decreased phospholamban phosphorylation (Fig. 4A) 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\textsuperscript{17} phosphorylation (Fig. 4B). Note that the isoproterenol-induced increase in Ser\textsuperscript{16} phosphorylation was the same at any of the [Ca\textsubscript{o}] assayed. Under conditions of extremely low Ca\textsuperscript{2+} supply to the myocardium, in which Thr\textsuperscript{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 32P at two different [Ca\textsubscript{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\textsubscript{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\textsubscript{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\textsubscript{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 32P/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. 6A) showed that the increase in [Ca\textsubscript{o}] did not increase phosphorylation of either Thr\textsuperscript{17} or Ser\textsuperscript{16} residues. The same increase in [Ca\textsubscript{o}] in the presence of 1 μM OA increased phosphorylation of Thr\textsuperscript{17} without affecting Ser\textsuperscript{16} (Fig. 6A).
enables the use of phospholamban since no cross-reactivity with the other site of phosphorylation was observed. The combination of this discrimination between the two sites of phosphorylation of phospholamban and the apparent discrepancy between the activation of 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, i.e., phosphatase inhibitor, failed to detect the unphosphorylated protein and proved to be highly specific in the absence of intracellular Ca2+ 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 Ca2+ evoked by cAMP-dependent mechanisms are large enough to activate CaMKII pathway. 2) CaMKII cascade could be activated only by compartmentalized increases in Ca2+ evoked by cAMP increases. 3) CaMKII could be activated by cAMP-dependent mechanisms unrelated to intracellular Ca2+ increase. 4) Phosphatases that dephosphorylate phospholamban could be inhibited by a PKA-dependent mechanism. The fact that increases in contractility (reflecting cytosolic Ca2+) 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 Thr17 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 Thr17 residue in the intact heart could be

| Treatment | Maximal rate of contraction % of control | Half relaxation time Δ msec | cAMP 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* (10) | −10.75 ± 0.90* (10) | 1.097 ± 0.036* (3) |
| 30 nM 1.35 mM [Ca]o | 172.42 ± 7.01* (9) | −19.95 ± 1.78* (19) | 1.696 ± 0.054* (6) |
| 300 nM 1.35 mM [Ca]o | 187.52 ± 21.17* (10) | −19.11 ± 0.56* (10) | 1.759 ± 0.204* (3) |
| 30 nM 0.25 mM [Ca]o | 106.05 ± 8.80 (9) | −9.67 ± 1.69* (9) | 1.692 ± 0.096* (3) |
| 30 nM 0.07 mM [Ca]o + 400 nM Nife | ND | ND | 1.610 ± 0.178* (3) |
| Calcium 3.85 mM [Ca]o | 135.57 ± 8.91* (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* (7) | −8.57 ± 3.87 (7) | 0.759 ± 0.142 (3) |

* p < 0.05 when compared to control.
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. 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\_] 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 (PStd\(_{16}\)-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."
inhibited by OA (18). We have found that increases in \([\text{Ca}]_o\), 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 \(\text{Ca}^{2+}\) 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 PPI 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 PPI activity (22).

For several years, different laboratories including our own, attempted to define the physiological role of the CaMKII pathway of phosphorylation of Thr\(^{17}\) residue of phospholamban. 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 \(\beta\)-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 \(\beta\)-adrenergic stimulation, activation of CaMKII cascade accounts for about 50% of phos-
Phospholamban phosphorylation. This CaMKII-induced phospholamban phosphorylation is closely associated with an increase in the relaxant capacity of the intact ventricle.

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