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Characterization and Quantitation of Phospholamban and Its Phosphorylation State Using Antibodies

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Quantitative immunoassays to discriminate and quantitate phospholamban and its phosphorylation states in heart homogenates were developed using known amounts of protein determined by amino acid analysis. Synthetic 1-52 phospholamban, the hydrophilic 1-25 peptide, and 1-25 phosphopeptides containing P-Ser^{16}, P-Thr^{17}, and dually phosphorylated (P-Ser^{16}, P-Thr^{17}) were used to calibrate immunoblot systems. In addition, synthetic 1-52 peptide was phosphorylated using cAMP-dependent protein kinase (P-Ser^{16}) or Ca^{2+}-calmodulin protein kinase (P-Thr^{17}) and then separated from unphosphorylated 1-52 by HPLC prior to quantitation. Further, canine cardiac sarcoplasmic reticulum was phosphorylated *in vitro* using [γ-^{32}P]-ATP with cAMP-dependent protein kinase and/or Ca^{2+}-calmodulin-dependent protein kinase as well as sequential phosphorylation in both orders to assess the veracity of antibody recognition of phosphorylated forms. Western blots proved useful in characterizing the reactivity of the different antibodies to phospholamban and phosphorylated phospholamban, but were inefficient for accurate quantitation and problems with antibody recognition of dually phosphorylated phospholamban were found. mAb 1D11 recognized all forms of phospholamban, polyclonal antibodies 285 and PS-16 were highly selective for P-Ser^{16} phospholamban but had diminished reactivity to diphosphorylated (P-Ser^{16}, P-Thr^{17}) phospholamban, and polyclonal antibody PT-17, although selective for P-Thr^{17} phospholamban, generated very weak signals on Western blots and reacted poorly with diphosphorylated phospholamban. Results in quantitative immunodot blot experiments were even more compelling. None of the phosphorylation specific antibodies reacted with the diphospho 1-25 phospholamban peptide. Transgenic mouse hearts expressing varying levels of PLB and ferret heart biopsy samples taken before and after isoproterenol perfusion were analyzed. In all samples containing phospholamban, a basal level of Ser^{16} phosphorylation (about 4% of the total PLB population) and a lesser amount of Thr^{17} phosphorylation was observed. Upon isoproterenol perfusion, Ser^{16} phosphorylation increased only to 17% of the total phospholamban population with a similar change in Thr^{17} phosphorylation. This suggests that phospholamban phosphorylation may serve as an electrostatic switch that dissociates inactive calcium pump complexes into catalytically active units. Thus, direct correlations between phospholamban phosphorylation state and contractile parameters may not be valid.

Key Words: **phospholamban; phosphorylation; phosphorylation specific antibodies.**

The prominence of its phosphorylation upon β-adrenergic stimulation of heart led to the discovery and nomenclature of phospholamban (PLB) (Tada *et al.*, 1974). It is widely accepted that phosphorylation of PLB by cAMP-dependent or Ca^{2+}-calmodulin-dependent protein kinases relieves PLB's inhibitory effect on Ca^{2+}ATPase leading to an increased calcium sensitivity of the sarcoplasmic reticulum calcium pump. The precise mechanism of this inhibition is not known. Several reports (Wegener *et al.*, 1989; Napolitano *et al.*, 1992; Talosi *et al.*, 1993) correlate increases in PLB phosphorylation with cardiodynamics and demonstrate sequential phosphorylation at Ser^{16} followed by Thr^{17} *in vivo.* Independent phosphorylation occurs *in vitro* at Ser^{16} by cAMP-dependent protein kinase and Thr^{17} by Ca^{2+}-calmodulin-dependent protein kinase (Simmerman and Jones, 1998, review). Phosphorylation at either site can alleviate PLB's inhibitory effect on calcium sensitivity and may cause an increase in myocardial contractility and faster relaxation (Mundina-Weilenmann, 1996) with maximal effects occurring when both sites become phosphorylated.

Numerous reports (Hasenfuss, 1997, review) demonstrate reductions in PLB expression in failing hearts, yet no one has accurately quantitated the protein ex-

Abbreviations used: mAb, monoclonal antibody; P_i, inorganic phosphate; PLB, phospholamban; SR, sarcoplasmic reticulum; PBS, phosphate-buffered saline; BSA, bovine serum albumin.

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pression level nor the phosphorylation state(s) of PLB in any preparation. The ability to accurately quantitate PLB and its phosphorylation state from small tissue samples would contribute to clarifying the role, if any, that this regulatory protein plays in the pathogenesis of heart failure. The report (Drago and Colyer, 1994) that phosphorylation specific antibodies (PS-16 and PT-17) could be raised against PLB presented an opportunity to develop assays to determine and quantitate the different phosphorylation states of PLB. These assays could then be applied to the study the role of PLB phosphorylation during inotropic intervention and the analysis of samples from disease models and human heart failure biopsies. An immunoblot assay to quantitate PLB in crude heart homogenates using mAb 1D11 (Mayer *et al.*, 1996) with synthetic PLB for calibration has been described (Mayer *et al.*, 1998). This assay measures the total amount of PLB regardless of its phosphorylation state and allows the handling of a large number of samples. This report extends the application with mAb 1D11 to incorporate the results using polyclonal Ab 285 which is selective for P-Ser^{16} PLB and compares them with polyclonal antibodies PS-16 and PT-17 (Drago and Colyer, 1994). In addition, problems with sample handling are presented indicating some limitations of the utility of these probes for assessing complete phosphorylation events.

EXPERIMENTAL PROCEDURES

Materials. Phospholamban was synthesized using an Applied Biosystems 430A synthesizer. PLB 1-25 peptide and phosphopeptides were prepared by Research Genetics. Polyclonal antibody 285 was raised by Hazelton Research Products against PLB 9-19Y P-Ser^{16} (California Peptide Research) conjugated to keyhole limpet hemocyanin. Polyclonal antibodies PS-16 and PT-17 were purchased from PhosphoProtein Research. Secondary antibodies and alkaline phosphatase substrates were purchased from ICN. Phosphorylated synthetic phospholamban (1-52) was separated from non-phosphorylated protein by reverse-phase HPLC on a Toyoshima C_{18} column using a linear gradient of 13–23% acetonitrile, 0.1% trifluoroacetic acid over 20 minutes. Stock solutions of protein were subjected to total (100 h) acid hydrolysis and amino acid analysis on INSTRUMENT. The veracity of the phosphorylated peptides was confirmed by analysis on a MODEL mass spectrometer. Sarcoplasmic reticulum vesicles were prepared from canine heart as described by Krause and Hess (1984).

Phosphorylation of synthetic phospholamban and cardiac sarcoplasmic reticulum. Protein (0.5 mg/ml) were phosphorylated in a final volume of 250 μl of buffer comprised of 25 mM Histidine/HCl, pH 7.0, 10 mM $MgCl_2$, 0.5 mM EGTA, 0.475 mM $CaCl_2$ (10 μM free), and 10 mM NaF. For phosphorylation at Ser^{16}, 10 units of catalytic subunit of cAMP-dependent protein kinase (Sigma) was included in the buffer, while for phosphorylation of Thr^{17}, 1 μM calmodulin (Sigma) was included to activate the endogenous sarcoplasmic reticulum Ca^{2+}-calmodulin-dependent protein kinase or 0.72 units of Ca^{2+}-calmodulin kinase II (Calbiochem) was added for synthetic PLB. All phosphorylations were initiated by addition of 0.5 mM [γ-^{32}P]-ATP. For sequential phosphorylation, the catalytic subunit or calmodulin was added 2 minutes after initiation of the first reaction. After 10 minutes, all reactions were stopped with 2× SDS–PAGE sample buffer (100 mM Tris/HCl, pH 6.8, 4% SDS, 24% glycerol, 100 mM DTT, and bromphenol blue dye), boiled, and run on acrylamide gels. Gels were transferred onto 0.05 μm nitrocellulose membranes (Schleicher & Schuell) for Western blot analysis and incorporated radioactivity was measured using a Molecular Dynamics Phosphorimager.

Modified dot blot. Peptides (0.01 ng–10 μg) were pipetted (5 μl) onto 0.05 μm nitrocellulose membranes and allowed to dry overnight. Non-specific binding was minimized by blocking with 3% BSA in 20 mM Tris/HCl, pH 7.4, 150 mM NaCl (TBS) for 1 h at room temperature. The membrane was then rinsed two times in TBS containing 0.05% Tween 20 for 1 h. PLB was visualized by treating the membrane with 25 ml of 9.6 μg/ml mAb 1D11 or a 1/1000 dilution of Ab 285 for three hours followed by three rinses with TBS/Tween. This was followed by the addition of a 1/1000 dilution of secondary antibody (goat anti-rabbit IgG or sheep anti-mouse IgG) conjugated to alkaline phosphatase for two hours followed by four rinses with TBS/Tween. Spots were visualized by placing the membranes in a solution of 0.1 M Tris/HCl, pH 9.5 containing nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate previously solubilized in dimethylformamide for 20 minutes then rinsing with H_2O. Multiple sample dilutions were analyzed in the linear range of the standard curve.

Western blots. Total protein was measured in crude cardiac homogenates using a modified Amido black assay (derived from Mahoney *et al.*, 1997) with BSA as the standard. All wash steps in the assay were changed to 50% methanol (150 μl) and eluted dye was transferred to a microtiter plate for absorbance reading. Homogenates were added to 2× SDS–PAGE sample buffer, boiled for 5 minutes and run on a 10–20% Tris/Tricine gel. Protein was then transferred onto a 0.05 μm nitrocellulose membrane using a semi-dry transfer apparatus (Millipore). Immunoreactive protein was visualized as described above.

In vivo phosphorylation of ferret hearts. Ferret hearts were perfused with oxygenated (95% O_2/5% CO_2) physiological saline solution (1.2 mM NaH_2PO_4, pH 7.4, 2.5 mM $CaCl_2$, 120 mM NaCl, 5.9 mM KCl, 11.5 mM glucose, 25 mM $NaHCO_3$, 1.20 mM $MgCl_2$) at 37°C using a Langendorff protocol. After reaching equilibrium which was determined visually by observing a steady rate of cardiac contraction, biopsies (approximately 10 mg in size) were cut from the apex of the ventricle and immediately freeze clamped or frozen in a bath of liquid N_2. For β-adrenergic stimulation, 10 μM isoproterenol was added to the perfusion media. Upon reaching a steady state of contractility, the remaining heart tissue was freeze clamped or placed into a bath of liquid N_2. Biopsies were homogenized under liquid N_2 with a pre-chilled mortar and pestle in the presence of solubilization buffer (50 mM Na PO_4, pH 7.0, 10 mM Na EDTA, 10 mM NaF, 50 mM *n*-octyl-β-D-glucopyranoside, 1 mM AEBSF and 1 mM DTT). Addition of solubilization buffer at this step had the dual advantages of increasing the volume of the biopsy, reducing losses due to adherence to the sides of vessels and ensuring that as soon as the homogenate is thawed it comes in contact with phosphatase and protease inhibitors. Upon thawing, solutions were centrifuged in a microfuge for 5 minutes at maximum speed, the supernatant was stored at −70°C.

RESULTS

Western Blot Analyses

The specificity of polyclonal antibody 285 for synthetic PLB and P-Ser^{16} synthetic PLB compared to mAb 1D11 is shown (Fig. 1). Typical for synthetic PLB, a ladder of bands persists even after boiling. This probably reflects the hydrophobic nature of pure PLB. The small change in molecular mass due to the addition of

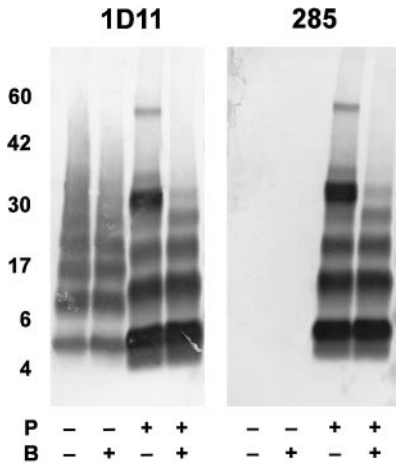

FIG. 1. Western blot analysis of synthetic phospholamban with antibodies 1D11 and 285. Equal amounts (100 ng) of synthetic phospholamban (first two lanes of each panel) and P-Ser[16] phosphorylated (P) phospholamban (last two lanes) were probed before (lanes 1 and 3) and after boiling (lanes 2 and 4, B) in SDS–PAGE sample buffer containing 10 mM DTT.

phosphate becomes more apparent as the oligomeric size increases, since the aggregation amplifies this small change in size. Similar results using PS-16 (not shown) were also obtained. The addition of phosphate to PLB appears to favor a pentameric complex and a higher order complex (see lane 3) which dissociate upon boiling. Both 285 and PS-16 had some cross-reactivity with synthetic P-Thr[17] PLB (data not shown). In spite of numerous attempts with PT-17, its specificity for P-Thr[17] synthetic PLB could not be conclusively demonstrated. Instead, PT-17 often reacted with P-Ser[16] PLB (data not shown).

Cardiac sarcoplasmic reticulum membranes were phosphorylated with [γ-^{32}P]-ATP *in vitro* using the catalytic subunit of cAMP-dependent protein kinase, the endogenous Ca^{2+}-calmodulin-dependent protein kinase, sequential phosphorylation in both orders, and simultaneous phosphorylation (Fig. 2). Samples were subjected to SDS–PAGE and Western blot analyses followed by autoradiography to determine ^{32}P incorporation. The specificity of mAb 1D11, polyclonal antibody 285, and polyclonal antibody PT-17 in Western blots for PLB and its phosphorylated forms are shown (top panel). The histograms (bottom panel) show that either kinase alone phosphorylates to nearly the same extent (lanes 2 and 3) and their effects were nearly additive when performed sequentially (lanes 4 and 5). Simultaneous reactions were not as effective (lane 6). mAb 1D11 elicited broad band signals of equal intensity for all phosphorylation conditions. Polyclonal antibody 285 produced thinner bands with slightly slower electrophoretic mobility. Surprisingly, roughly 5% of the total PLB under basal conditions (lane 1) consisted of P-Ser[16] PLB in the isolated sarcoplasmic reticulum preparation, even in the absence of phosphatase inhibitors during preparation. The signal intensity increased, about 5-fold following phosphorylation using cAMP-dependent protein kinase (lane 2). This increase was consistent with the measured increase in ^{32}P-incorporation. Following activation of Ca^{2+}-calmodulin-dependent protein kinase (lane 3), the signal intensity diminished substantially suggesting that the presence of high calcium and/or calmodulin may have activated a P-Ser[16] reactive phosphatase or inhibited an endogenous kinase responsible for the basal phosphorylation or that P-Thr[17] phosphorylation reduced the antigenicity. Sequential phosphorylation (lanes 4 and 5) and simultaneous phosphorylation (lane 6) yielded equivalent band intensities which were less than that obtained using cAMP-dependent protein kinase alone.

Antibody PT-17 detected a low level of basal P-Thr[17] PLB (lane 1) which did not change after cAMP-

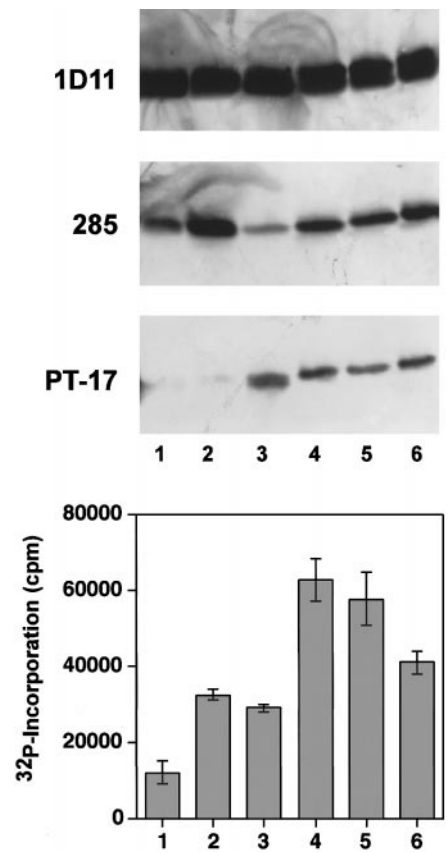

FIG. 2. Western blot analysis and autoradiography of phosphorylated cardiac sarcoplasmic reticulum samples. Canine cardiac sarcoplasmic reticulum was phosphorylated *in vitro* using [γ-^{32}P]-ATP as described in the methods. Protein (10 μg) was probed with mAb 1D11, antibody 285, and PT-17. ^{32}P-incorporation (CPM) was measured by phosphorimager quantitation.. The samples were mock phosphorylation (no exogenous cAMP-dependent protein kinase or calmodulin or Ca^{2+}) (lane 1), cAMP-dependent protein kinase (lane 2), Ca^{2+}-calmodulin-dependent protein kinase (lane 3), cAMP-dependent protein kinase followed by Ca^{2+}-calmodulin-dependent protein kinase (lane 4), Ca^{2+}-calmodulin-dependent protein kinase followed by cAMP-dependent protein kinase (lane 5), and simultaneous phosphorylation (lane 6).

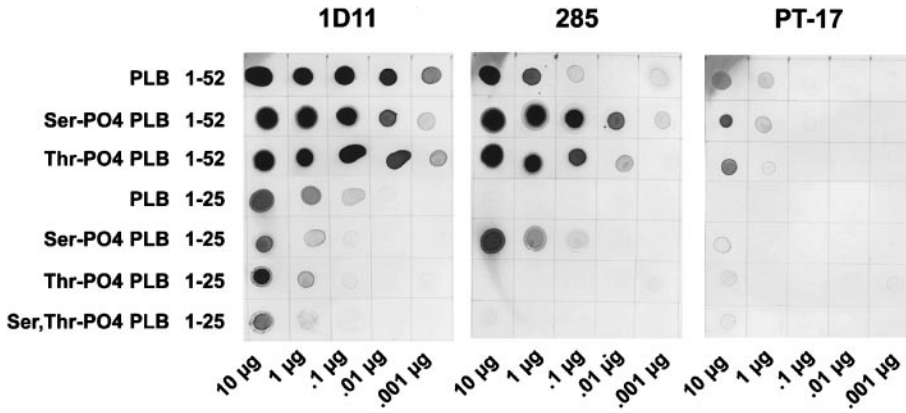

FIG. 3. Dot blot analysis of synthetic phospholamban and 1-25 phospholamban peptide. All forms of phospholamban were examined in logarithmic increments ranging from 10 μg to 10 ng protein. The top panel shows synthetic PLB and its monophosphorylated forms. The bottom panel shows the 1-25 PLB peptide and its three phosphorylated forms. Amido black staining and quantitation shows that equivalent amounts of each form of protein bound to the membrane at least in the range of 0.1 to 10 μg. The panels for mAb 1D11, antibody 285, and PT-17 are shown. PS-16 was tried, but a very low signal intensity was detectable at 10 μg protein (not shown).

dependent protein kinase activation (lane 2). *In vitro* phosphorylation using the endogenous Ca^{2+}-calmodulin-dependent protein kinase yielded the strongest band (lane 3) which had slightly slower electrophoretic mobility and thinner width than observed with mAb 1D11. Sequential phosphorylation reactions (lanes 4 and 5) and simultaneous reactions (lane 6) yielded bands of reduced intensity.

These results can not determine whether dual phosphorylation occurred or if different populations of PLB were phosphorylated by each kinase. Even with pure synthetic PLB and efforts to drive the phosphorylation reactions to completion, at best only about 80% of the PLB could be phosphorylated. The histogram from autoradiography suggests that the sequential reactions were close to being additive, thus, decreases in signal intensity should not occur. The simultaneous reactions had reduced incorporation of radioactive phosphate and antibody 285 indicated that near maximal incorporation onto Ser^{16} occurred, therefore, substantially reduced levels of radioactive phosphate onto Thr^{17} was expected. This was not observed. Examination of the bands on the mAb 1D11 Western blots before and after phosphorylation reaction suggests that a large percentage of PLB remained non phosphorylated. The bands are much broader and exhibit some distortion toward the top of the gel upon phosphorylation indicative of an increase in population of a slower moving form. Thus, all of these conditions represent a heterogeneous population of PLB.

Dot Blot Analyses with Synthetic Samples

Pilot experiments indicated that the PLB 1-25 peptide bound poorly to 0.2 μm nitrocellulose membranes using a conventional vacuum-driven dot blot apparatus. Better results were obtained by directly applying the sample onto tighter pored, 0.05 μm nitrocellulose and allowing the sample to air dry. Multiple sets of serially diluted samples (derived from a stock solution calibrated by amino acid analysis and authentication of phosphorylation state by mass spectral analysis) were applied and after blocking with bovine serum albumin were probed with the different antibodies. To calibrate the assay, the highly soluble, PLB 1-25 peptide and its well-defined phosphorylated forms were analyzed. A set of samples of 1-25 peptide were applied to a separate membrane and subjected to Amido black staining. The Amido black assay which is sensitive to protein amounts ranging from 0.1 to 10 μg confirmed that the majority of the protein adhered to the membrane and all the samples behaved similarly. This finding was confirmed by the results with mAb 1D11 (Fig. 3, left) which gave nearly identical standard curves for each form of the 1-25 peptide. Despite being roughly half the size of PLB, the detection of the 1-25 peptide was over 100 times less than full-length PLB. The 1-25 peptides were detectable in the range of 100 ng to 10 μg. Antibody 285 was highly selective for the Ser^{16} 1-25 phosphopeptide compared to the unphosphorylated peptide, Thr^{17} phosphopeptide, and Ser^{16}-Thr^{17} diphosphopeptide (Fig. 3, middle, lower portion) and exhibited a similar sensitivity range with mAb 1D11. PS-16 (data not shown) had a similar recognition profile but low signal strength. Importantly, no signal was obtained, even at 10 μg, with Ser^{16}, Thr^{17} diphosphopeptide. Lastly, PT-17 (Fig. 3, right) had poor sensitivity and non-selectively bound to all of the phosphorylated forms of 1-25 peptide, but not to the non phosphorylated peptide.

Full-length PLB elicited greater immunoreactivity than the 1-25 peptide with all of the antibodies tested. Like with the 1-25 peptides, stock solutions of syn-

thetic PLB and PLB after phosphorylation reactions and HPLC separation were calibrated using amino acid analysis. Attempts to distinguish the phosphorylation states using MALDI and Microspray LC/MS were unsuccessful. It was assumed that the correct phosphorylation sites were used by the two kinases. Despite applying roughly half the molar amount, the antibody sensitivities were more than 1000 times higher. The sensitivity range for mAb 1D11 ranged from 0.1 ng to 1 μg and, again, mAb 1D11 does not discriminate among the various phosphorylated states. Antibody 285 had a sensitivity range form 1 ng to 1 μg and exhibited greater than 100 fold selectivity for P-Ser[16] PLB versus non-phosphorylated PLB. It had only about a 10 fold selectivity versus P-Thr[17] PLB. PS-16 (data not shown) elicited a very weak signal when 10 μg PLB was probed and did not seem to discriminate among the phosphorylated forms. Like with the 1-25 peptides, PT-17 was somewhat selective for monophosphorylated forms of full length PLB, but poorly discriminated among the two forms. The exact authenticity and purity of the dually phosphorylated synthetic PLB could not be verified and, therefore, this sample could not be included in the antibody characterization. In addition to getting antibody 285 prepared, attempts to raise antibodies selective for Thr[17] phospho-PLB and Ser[16], Thr[17] diphospho-PLB were unsuccessful. Several antibodies were raised yielding stronger signals than PS-16 and PT-17 on dot blots, but they failed to exhibit the proper selectivity (data not shown).

Quantitation of Phospholamban and Phosphorylated Forms

While these antibodies have tremendous potential for discriminating the different phosphorylated PLB forms, they failed to fully achieve this goal. We previously reported (Mayer *et al.*, 1998), the use of mAb 1D11 to quantitate the total amount of PLB in small cardiac samples. We had hoped to extend these quantitations to decipher the phosphorylation state(s) of PLB using phosphorylation selective polyclonal antibodies. As the previous sections indicate, these antibodies have selectivity for mono-phosphorylated PLB forms over the non phosphorylated protein and do not appear to react with di-phosphorylated PLB.

As a test of the ability to use these antibodies for quantitation, ferret heart homogenates were analyzed for PLB and P-Ser[16] PLB levels before and after β-adrenergic stimulation using 10 μM isoproterenol. Since the purpose of these experiments was to assess the ability of these antibodies to discriminate and quantitate the various phosphorylated PLB forms, no correlation to contractile parameters were attempted and all samples were boiled to drive the PLB into a monomeric state. Control samples were excised from the left ventricle and immediately frozen in liquid nitrogen. Three samples following isoproterenol perfusion were taken; excision from the left ventricle and immediately frozen, excision from the left ventricle and frozen after waiting 1 minute at room temperature, and freeze-clamping the remaining heart. The total PLB level as determined on dot blots using mAb 1D11 was 2.50 ng per μg of protein. For comparison, mouse heart homogenate contained 0.97 ng per μg of protein. Assuming phospholamban's molecular weight to be 6123 g/mole, then the total phospholamban level in ferret heart is 0.408 nmol/mg protein. Employing antibody 285, the basal Ser[16] phosphorylation level was about 0.1 ng P-Ser[16] phospholamban per μg of protein (approximately 4% of the total). Following β-adrenergic stimulation and freeze clamping, the P-Ser[16] PLB level increased to about 0.43 ng per μg protein (about 17% of the total phospholamban content) and the freezing in liquid nitrogen sample handlings resulted in lower levels. No detectable amounts of Thr[17] phosphorylation were found using antibody PT-17 by dot blot. Regardless of the contribution of P-Thr[17] PLB, a ~4.3-fold increase in Ser[16] phosphorylation state is consistent with measurements using ^{32}P-incorporation into PLB in guinea pig hearts (4.6-fold, Rapundalo *et al.*, 1989) and rat hearts (6-fold increase, Vittone *et al.*, 1998) or rat heart myocytes (Calaghan *et al.*, 1998b). In addition, the amount of phosphorylated PLB is consistent with the maximal ^{32}P incorporation of 0.278 ng per μg protein measured in rat heart homogenates following isoproterenol stimulation (Mundina-Wielenmann *et al.*, 1996).

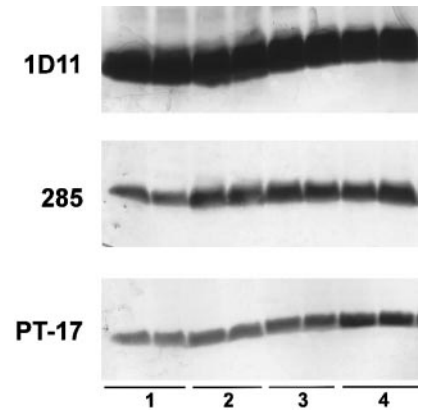

FIG. 4. Phosphorylation in ferret heart during β-adrenergic stimulation. Ferret heart homogenates run in duplicate (4 μg protein/well for mAb 1D11 and 20 μg/well for 285 and PT-17) were analyzed by Western blot analysis. Two sampling methods (excision and immersion into liquid nitrogen (set 3) and direct freeze clamping (set 4)) were compared to control (set 1, tissue was excised and rapidly frozen prior to isoproterenol perfusion). In addition, the effect of slow tissue handling, a 1 minute wait prior to immersion in liquid nitrogen (set 2) was examined.

Western blots (Fig. 4) were also performed on samples taken from perfused ferret hearts and the sample

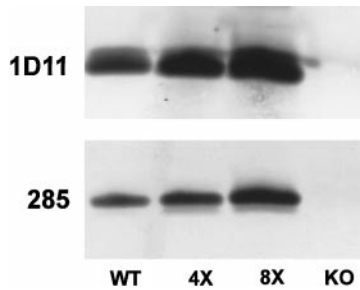

FIG. 5. Phospholamban levels and basal P-Ser[16] phospholamban levels in transgenic mouse hearts. Heart homogenates (1 μg protein/well and 10 μg/well for knock-out hearts) were prepared as described by Mayer *et al.*, 1998 from phospholamban knock-out (KO), wild-type (WT), heterozygous overexpressing (4X), and homozygous overexpressing (8X) mice. Western blot analysis was performed using mAb 1D11 and antibody 285. Synthetic phospholamban or P-Ser[16] synthetic phospholamban (not shown) was analyzed on each blot for approximating the amount of each form. In addition, quantitative immunoblot assays were also performed.

handling methods were compared. As shown with mAb 1D11, equal amounts of PLB were present in all samples. Basal P-Ser[16] PLB was detected by antibody 285 and the amount increased roughly 4-fold following isoproterenol intervention. Sample handling did not appear to alter the basal level of P-Ser[16] PLB (data not shown), but had pronounced effect on the end phosphorylation state following isoproterenol perfusion. Freeze-clamping was the only reliable method for preserving PLB phosphorylation due to isoproterenol perfusion. Marked loss of isoproterenol-induced PLB phosphorylation occurred, even when the excised tissue was immediately immersed into liquid nitrogen and within a minute nearly all the newly formed phosphorylated PLB has been dephosphorylated. The presence of 10 mM NaF in the homogenization buffer during thawing and homogenization is helpful in slowing phosphatase activity, but can not restore the lost phosphorylated PLB from tissue freezing. Although not quantitated using dot blots and PT-17, some basal P-Thr[17] PLB was present on Western blots and roughly a 4-fold increase was observed. Only immediate freeze-clamping preserved the maximal increase in P-Thr[17]. The rapid dephosphorylation of PLB that occurs during sample freezing makes accurate assay of biopsy samples questionable.

The presence of a basal P-Ser[16] PLB population in all of the samples examined was intriguing. Cardiac homogenates were prepared from transgenic mice expressing no phospholamban ranging to 8.8 fold overexpression relative to wild-type mice (Mayer *et al.*, 1998). Samples were subjected to Western blot analysis (Fig. 5) and probed with mAb 1D11 and antibody 285. In agreement with dot blot quantitations ($\pm$SEM) of 0.00 ng PLB/μg protein (knock-out), 0.97 $\pm$ 0.13 (wild-type), 3.99 $\pm$ 0.75 (heterozygous overexpression), and 8.80 $\pm$ 1.39 (homozygous overexpression), the total amount of phospholamban detected by mAb 1D11 increased in similar increments; 0, 1, 3, 8 ng/μg protein, respectively. At the same time, the amount of phosphorylated PLB detected by antibody 285 increased in parallel suggesting that the heart maintains a fixed portion ($\sim$5%) of the total population as P-Ser[16] PLB.

DISCUSSION

The results, using 1-25 phosphoPLB peptides demonstrate that phosphorylation site-specific antibodies (285, PS-16, and PT-17) can discriminate between Ser[16] and Thr[17] phosphorylation and presumably of full-length PLB in cardiac SR on Western blots as previously reported (Drago and Colyer, 1994; Mundina-Wielenmann *et al.*, 1996; Vittone *et al.*, 1998). The best results were obtained when low amounts of synthetic PLB (less than 0.1 μg) were used. mAb 1D11 reacted comparably with all forms of PLB as well as with all 1-25 PLB peptides, although with diminished sensitivity. Polyclonal antibody 285 is highly selective for P-Ser[16] PLB and performed better than PS-16 on immunodot blots in both signal intensity and at discriminating between P-Ser[16] and P-Thr[17] PLB. When the chemically synthesized diphosphorylated 1-25 PLB peptide was probed, little if any antibody 285 reactivity was found. Although not shown, PS-16 also did not react with the diphosphorylated 1-25 PLB peptide. A brief note of unpublished data (Calaghan *et al.*, 1998b) alludes this lack of immunoreactivity by PS-16. PT-17 elicited low signal strength and exhibited poor selectivity on immunodot blots among all forms of PLB and could only discriminate against non-phosphorylated 1-25 PLB peptide. Attempts to raise novel antibodies specific for P-Thr[17] and P-Ser[16], P-Thr-[17] phospholamban were unsuccessful. We had hoped to obtain high throughput assays to accurately quantitate PLB levels and each distinct phosphorylation state to serve as a standard for studies to assess the potential roles of PLB phosphorylation during inotropic intervention and the pathogenesis of heart disease. The literature is severely lacking in such numbers and investigators have been satisfied with the use of arbitrary units and relative comparisons. As the results indicate, all the required techniques have not been fulfilled, due to problems with antibodies sensitivity and selectivity. In addition, problems with preserving the phosphorylation state during sample handling needs to be solved.

The availability of synthetic PLB and precise phosphorylated forms of 1-25 PLB peptide allows the calibration of immunoassays. As questioned by Holzhauer, 1987, quantification of PLB by measuring the *in vitro* incorporation of ^{32}P-phosphate does not provide an accurate measure of total PLB nor allow the determination of basal phosphorylation state. Indeed, even with synthetic PLB and conditions favoring phosphoryla-

tion, at best only 80% phosphorylation occurred using the catalytic subunit of cAMP-dependent protein kinase (Mayer *et al.*, 1996). Dual phosphorylation was even less efficient. While the hydrophilic 1-25 peptide and chemically synthesized phosphopeptides proved useful for assessing immunoreactivity and antibody selectivity, full-length PLB yielded a higher sensitivity range of immunodetection. Standard curves were constructed in immunodot blot experiments as previously reported (Mayer *et al.*, 1998) using mAb 1D11 allowing quantitation of total PLB from various cardiac samples. Since antibody 285 worked well with full-length PLB, a calibration curve was constructed to quantitate P-Ser^{16} PLB forms from small tissue samples. Due to low signal strength, a standard curve for P-Thr^{17} PLB and polyclonal Ab PT-17 could not be constructed.

Unexpectedly, the phosphospecific antibodies exhibited diminished immunoreactivity with samples where conditions favoring dual phosphorylation were performed. The data with the well-characterized 1-25 phospho-PLB peptides suggest that the antibodies are capable of discerning mono-phosphorylated PLB from the di-phosphorylated PLB. Thus, the diminished immunoreactivity may reflect the formation of di-phosphorylated PLB from mono-phosphorylated PLB, although activation of a secondary event, e.g., P-Ser phosphatase, can not be excluded. A diminished level of basal P-Ser^{16} PLB was always observed following calcium elevation and calmodulin addition to canine cardiac SR. Several reports (Wegener *et al.*, 1989; Napolitano *et al.*, 1992; Talosi *et al.*, 1993) demonstrated sequential phosphorylation of Ser^{16} followed by Thr^{17}. This problem may account for the unexpected decrease in Ser^{16} phosphorylation observed by Vittone *et al.* (1998) when isoproterenol was used at high $[Ca]_o$. The authors could not account for an observed decrease in phosphorylation level.

Although additivity of phosphorylation of PLB between cAMP-dependent and Ca^{2+}-calmodulin-dependent protein kinases does occur, it is unclear whether separate PLB subunits each gets phosphorylated or the same PLB subunit receives both phosphates. It appears that, during β-adrenergic stimulation, roughly one out of five PLB molecules gets phosphorylated by each kinase. The Western blots suggest that PLB phosphorylation stabilizes a pentameric complex consistent with mutagenesis experimentation and polymerization model (Kimura *et al.*, 1997). Dual phosphorylation of the 1-24 PLB peptide was studied (Karczewski *et al.*, 1997). Phosphorylation of this putative monomeric 1-24 peptide, unlike PLB, occurred independently. When incubated with isolated cardiac SR, endogenous cAMP-dependent and Ca^{2+}-calmodulin-dependent protein kinase activities were found which may account for the presence of basal phosphorylated PLB. Although additive phosphorylation of 1-24 PLB peptide between cAMP-dependent and Ca^{2+}-calmodulin-dependent protein kinase was measured, the order of phosphorylation, the completeness of phosphorylation, and dual phosphorylation of a single peptide were not ascertained.

The basal phosphorylation state of phospholamban appears to vary dramatically among laboratories which may reflect the phosphatase activities, sample handling, pH, and extracellular calcium concentration during heart homogenization and preparation of membrane fractions. While low levels of basal P-Ser^{16} PLB were always measurable in canine SR preparations, mouse heart and ferret heart homogenates, significant reduction of β-adrenergic stimulated P-Ser^{16} PLB levels in ferret occurred if rapid freeze-clamping was not employed. It has been noted (Boateng *et al.*, 1997) that significant dephosphorylation occurs during tissue homogenization in the absence of phosphatase inhibitors and that it is vital to clamp the endogenous kinases and phosphatases during sample preparation in order to assess PLB phosphorylation status (Calaghan *et al.*, 1998a). Our results clearly show that rapid freeze clamping is the surest method to clamp the PLB phosphorylation state as dephosphorylation occurs after just a short time during tissue handling. Again, to stress the issue, ferret heart has highly active phosphatase activity which requires harsh conditions to control during initial homogenization. Complete dephosphorylation of basal P-Ser^{16} PLB was not seen. Examination of P-Ser^{16} PLB levels in transgenic mouse hearts expressing variable levels of PLB suggest that myocytes actively maintain a low population level (about 5%) of P-Ser^{16} PLB. No detectable basal levels of P-Thr^{17} were found in these species. Hulme *et al.* (1997) suggest that basal P-Thr^{17} phospholamban levels are up to 5 times higher than P-Ser^{16} phospholamban levels in rat myocytes. In contrast, no detectable basal levels of P-Thr^{17} phospholamban were detected in rat hearts (Mundina-Wielenmann *et al.*, 1996; Vittone *et al.*, 1998), and while not quantitated, variable low levels of P-Ser^{16} PLB are apparent throughout their reports.

A strong association between changes in cardiac dynamics induced by β-adrenergic stimulation and PLB phosphorylation are present. However, the precise relationship is only conjecture and speculation. Remarkably, although Ser^{16} phosphorylation occurs, the overall change (4 to 17% of the total PLB) was small and the majority of PLB remained non-phosphorylated. Concurrently, dramatic changes in cardiodynamics occurred. This implies that there is a large reserve pool of PLB and that phosphorylation of a small percentage of the total pool acts as an electrostatic switch as suggested by Fleischer (1994) allowing the recruitment (McKenna *et al.*, 1996) of aggregated, inactive calcium pumps into active units. Indeed, as more results from studies on PLB knock-out mice (Li *et al.*, 1998) and transgenic mice with non phosphorylatable PLB (Luo *et al.*, 1998) are obtained, the importance of PLB phos-

phorylation as protein kinase substrate is being reassessed. As we have learned, the overall changes in PLB phosphorylation status are relatively small and changes in contractile parameters occur in the absence of PLB or PLB phosphorylation. What's more, while large changes in contractile state occur with phosphodiesterase inhibition (Rapundalo *et al.*, 1989), very little or no changes in phosphorylation of SR and myofibrillar proteins were observed. Thus, while associations are attractive, clear-cut cause and effects are not evident.

While many have been tempted to correlate PLB phosphorylation (Mattiazzi *et al.*, 1994; Bassani *et al.*, 1995) or anti-PLB antibody (Sham *et al.*, 1991) with specific parameters of contractility and relaxation, myocytes appear to be too complicated with compensatory systems for simplistic cause and effects. For example, Thr^{17} phosphorylation has been suggested to contribute to the enhancement of relaxation observed in acidosis (Hulme *et al.*, 1997; Vittone *et al.*, 1998), yet significant acceleration of relaxation occurs in PLB knock-out heart (Li *et al.*, 1998) which is attenuated by KN-93, a CaMKII inhibitor. A note of caution when interpreting the action of KN-93, we (Coll *et al.*, 1998) found that KN-93 has direct inhibitory effects on Ca^{2+}ATPase with an IC_{50} of 10 μM. Lastly, although the basal parameters and responsiveness to isoproterenol were lower in mouse hearts expressing mutant Ser^{16}-Ala PLB (i.e., PLB inhibition is present but not reversible by Ser^{16} phosphorylation), significant shortening of relaxation time was observed (Luo *et al.*, 1998) and no detectable P-Thr^{17} was found following isoproterenol treatment.

We have successfully used these antibodies to examine dephosphorylation in cardiac sarcoplasmic reticulum following cAMP-dependent protein kinase phosphorylation to show that potential "phospholamban inhibitor compounds" (Coll *et al.*, 1999) do not inhibit phosphatase activity, while known phosphatase inhibitors like NaF and okadaic acid had pronounced retarding effects on the dephosphorylation. Again, some basal P-Ser^{16} PLB remained detectable in all samples.

While the availability of phosphoselective antibodies hold great potential for exploiting to develop quantitative assays for each form of PLB, in practice, many problems were encountered. Others (e.g., Wegener and Jones, 1984; Gasser *et al.*, 1986; Imagawa *et al.*, 1986; Li *et al.*, 1990; Jackson and Colyer, 1996) have tried to take advantage of the slight shift in electrophoretic mobility of the different forms (non-, mono-, and diphosphorylated) of PLB, but the best effects are observed with the pentameric complex. Surprisingly, no one has tried to exploit the large differences in isoelectric points; Jones *et al.* (1985) report values for nonphosphorylated PLB (p*I* 10) and P-Ser^{16} phosphorylated (p*I* 6.7) while Gasser *et al.* (1986) reports values for P-Ser^{16} PLB (p*I* 6.2), P-Thr^{17} PLB (p*I* 6.4), and P-Ser^{16}-P-Thr^{17} (p*I* 5.2).

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