Comparison of the Kinetic Effects of Phospholamban Phosphorylation and Anti-phospholamban Monoclonal Antibody on the Calcium Pump in Purified Cardiac Sarcoplasmic Reticulum Membranes†

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Received May 12, 1997; Revised Manuscript Received July 23, 1997⊗

ABSTRACT: Protein kinase A- (PKA-) catalyzed phosphorylation of phospholamban (PLN), the protein regulator of the cardiac Ca pump, mediates abbreviation of systole in response to β-adrenergic agonists. Investigators previously, however, have been unsuccessful in demonstrating an effect of PLN phosphorylation or anti-PLN monoclonal antibody (mAb), which is considered to mimic phosphorylation's well-known effect on $K_{m(Ca)}$, on microsomal Ca uptake at the (high) Ca^{2+} concentrations found intracellularly at peak systole. We therefore compared the effects of the catalytic subunit of PKA and anti-PLN mAb on the kinetics of Ca uptake in sucrose gradient-purified cardiac microsomes. Both treatments produced a 33−44% increase in $V_{max(Ca)}$ at 25 and 37 °C, and an 11−31% decrease in $K_{m(Ca)}$ with comparable changes in Ca^{2+}-ATPase activity. An acceleration of E_2P decomposition upon PLN phosphorylation may contribute to the increased $V_{max(Ca)}$ of Ca uptake at 25 °C but not at 37 °C, based on measurement of the kinetics of E_2P decomposition and steady-state E_2P formation from P_i at different temperatures. Our data document almost identical increases in $V_{max(Ca)}$ of microsomal Ca uptake with PLN phosphorylation or addition of anti-PLN mAb and hence provide insight into the kinetic mechanism of PLN's regulation of the cardiac sarcoplasmic reticulum Ca pump protein.

The physiological role of PLN,[1] the calcium pump regulator in cardiac SR membranes, has become well-established with the development of the PLN gene-deficient mouse (Luo *et al.*, 1994). PLN-deficient hearts exhibit mechanical properties similar to hearts from wild-type mice that have been maximally stimulated with β-adrenergic agonists. Thus in both cases one observes an increase in systolic left intraventricular pressure, stroke volume, and rates of pressure development and muscle relaxation and a decrease in diastolic and end diastolic left intraventricular pressure and time to peak tension, hence abbreviation of systole. The magnitude of these effects varies inversely with the amount of PLN expressed in the heart (Luo *et al.*, 1996).

On the basis of *in vitro* studies with microsomal preparations (Inui *et al.*, 1986; Kirchberger *et al.*, 1986; Suzuki & Wang, 1986), the physiological role of PLN can be interpreted in terms of an inhibitory function of PLN on the SR calcium pump and a release from inhibition upon phosphorylation of phospholamban by PKA in response to β-adrenergic agonists. Details of the molecular nature of the interaction of PLN with the calcium pump protein, however, are still relatively few. Almost all of the laboratories studying this problem have reported that PLN regulates $K_{m(Ca)}$ of calcium transport with little or no effect on $V_{max(Ca)}$ (Wray & Gray; 1977; Movsesian *et al.*, 1990; Kimura *et al.*, 1991; Morris *et al.*, 1991; Briggs *et al.*, 1992; Cantilina *et al.*, 1993; Luo *et al.*, 1994; Mattiazzi *et al.*, 1994; Reddy *et al.*, 1995, 1996; Toyofuku *et al.*, 1994; Odermatt *et al.*, 1996). Hence a change in $K_{m(Ca)}$ is now recognized by some investigators as the hallmark of PLN's regulation of the calcium pump (Kimura *et al.*, 1996). Recent kinetic data from our own laboratory, however, constitutes compelling evidence that PLN may regulate both $V_{max(Ca)}$ and $K_{m(Ca)}$ in cardiac microsomal preparations by acting on two or more steps in the catalytic cycle of the Ca^{2+}-ATPase (Antipenko *et al.*, 1997). In the latter study, we subjected cardiac microsomes to mild trypsin treatment, which cleaves the inhibitory PLN, thereby producing calcium pump activation. We had shown previously that the effects of this treatment on calcium pump kinetics are similar to those of phosphorylation of PLN by a protein kinase A that was partially purified from bovine heart (Kirchberger *et al.*, 1986). Critical to our demonstration that PLN affects both $V_{max(Ca)}$ of calcium uptake and $K_{m(Ca)}$ was the development of a preparation of purified cardiac vesicles that are functionally devoid of ruthenium red-sensitive calcium release channels. Since these channels are known to be activated by 2 μM and higher concentrations of Ca^{2+} (Meissner & Henderson, 1987), effects of PLN on $V_{max(Ca)}$ of calcium uptake measured at these Ca^{2+} concentrations may be blunted or obscured in most crude microsome preparations.

† This study was supported by Grants HL15764 (to M.A.K.) and DE10754 (to A.I.S.) from the National Institutes of Health and by a grant from The Procter and Gamble Company (to A.I.S.).

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⊗ Abstract published in *Advance ACS Abstracts,* October 1, 1997.

[1] Abbreviations: PLN, phospholamban; PKA, protein kinase A; SR, sarcoplasmic reticulum; mAb, monoclonal antibody; EGTA, ethylenebis(oxyethylenenitrilo)]tetraacetic acid; MES, 2-(*N*-morpholino)-ethanesulfonic acid; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; DMSO, dimethyl sulfoxide.

S0006-2960(97)01109-4 CCC: $14.00

Many of the laboratories that concluded that the main or only role of PLN is to regulate $K_{m(Ca)}$ of the calcium pump based their conclusion on the use of mAb against PLN (Movsesian *et al.*, 1990; Kimura *et al.*, 1991; Morris *et al.*, 1991; Briggs *et al.*, 1992; Cantilina *et al.*, 1993; Reddy *et al.*, 1995, 1996). Since similar decreases in $K_{m(Ca)}$ were observed with PKA and anti-PLN mAb in crude microsomes in an initial study (Morris *et al.*, 1991), it was concluded that anti-PLN mAb mimics PLN phosphorylation (*e.g.*, Cantilina *et al.*, 1993). It is presently unclear whether anti-PLN mAb can produce also an increase in $V_{max(Ca)}$ and hence mimic PLN phosphorylation in this respect as well as with respect to $K_{m(Ca)}$. Since a considerable body of literature has been published to indicate that anti-PLN antibody produces no change in $V_{max(Ca)}$ of calcium uptake (Movsesian *et al.*, 1990; Kimura *et al.*, 1996; Morris *et al.*, 1991; Briggs *et al.*, 1992; Cantilina *et al.*, 1993; Reddy *et al.*, 1995, 1996), we sought to determine whether the reported lack of an increase in $V_{max(Ca)}$ in response to anti-PLN mAb is ascribable to the presence of calcium release channels in crude microsome preparations, an intrinsic inability of the antibody to produce such an effect, or yet other reasons. In the present study, we compare the effects of anti-PLN mAb and the catalytic subunit of PKA on calcium pump kinetics in purified cardiac SR vesicles. We present data to demonstrate that (1) anti-PLN mAb and phosphorylation lead to almost identical changes in both $K_{m(Ca)}$ and $V_{max(Ca)}$ of the calcium pump and (2) phosphorylation of microsomes (*i.e.*, PLN) results in an increase in the rate of breakdown of the phosphorylated Ca^{2+}-ATPase intermediate (E_2P), which may contribute to the increase in $V_{max(Ca)}$.

EXPERIMENTAL PROCEDURES

Materials. Anti-PLN mAb was purchased from Affinity Bioreagents. Okadaic acid (free acid or sodium salt), thapsigargin, and the catalytic subunit of PKA were obtained from Sigma. Since the PKA catalytic subunit and mAb preparations contain salts and other reagents, control solutions were prepared on the basis of information supplied by the vendor. For the PKA catalytic subunit, the following stock control solution was prepared: 50 mM sucrose, 32 mM KH_2PO_4, 1.7 mM mercaptoethanol, 0.83 μM EDTA (disodium salt), and 0.22 mM dithiothreitol. This stock solution was diluted with a solution of 6 mg/mL dithithreitol by an amount that took into account the amount of nonprotein solids relative to the specific activity of the enzyme in different enzyme lots. The calculated concentrations of nonprotein solids in the control solution, in the specified relative concentrations, were equivalent to the concentrations of the nonprotein solids in the stock solution of PKA after reconstitution with a solution containing 6 mg/mL dithiothreitol. Typically, the various solids in the control solution were present in the final reaction mixture at 0.4 times the concentrations listed above when the PKA catalytic subunit concentration was 160 units/mL. The control solution for the stock solution of anti-PLN mAb consisted of phosphate-buffered saline containing 74 mM 3-(*N*-morpholino)propanesulfonic acid, 18 mM glycine, and 0.05% NaN_3. This solution was diluted further in the final reaction mixture to 1.4% of specified concentrations when the mAb concentration was 7.5 μg of protein/mL. All other reagents were obtained as described previously (Antipenko *et al.*, 1997).

Preparation of Microsomes. Crude cardiac microsomes, prepared from canine left ventricle, were purified on a sucrose step gradient (Antipenko *et al.*, 1997). Crude and purified microsomes were stored in liquid nitrogen. Unless indicated otherwise, protein was estimated by the biuret procedure with bovine serum albumin as the standard.

Calcium Uptake Assay. Calcium uptake was assayed in a standard reaction mixture that included 1 mM ATP, an ATP-regenerating system, 2 mM $MgCl_2$, 1 μM okadaic acid, a $CaCl_2$−EGTA buffer system (described below), and either 2.5 or 5.0 mM oxalate-Tris when the reaction was run at 25 or 37 °C, respectively (Antipenko *et al.*, 1997). The pH of all additions to the reaction mixture was adjusted, whenever appropriate, so as to result in a final pH of 6.8 at 25 or 37 °C. The final concentration of DMSO, the vehicle in which the okadaic acid was dissolved, was 0.5% and had no effect on calcium uptake or Ca^{2+}-ATPase activity (see below). At 25 °C, the final microsome concentration was 5 μg/mL and reactions were stopped by filtration after 2 and 4 min at all Ca^{2+} concentrations except 2, 4, and 9 μM, in which case reactions were stopped at 1 and 2 min. When testing effects of PKA, microsomes were added to the temperature-equilibrated reaction mixture containing 160 units/mL (final concentration) of the catalytic subunit of the enzyme, and after a 2-min incubation, a $CaCl_2$−EGTA buffer solution containing ^{45}Ca was added to start the calcium uptake reaction. The $CaCl_2$−EGTA buffer contained 125 μM $CaCl_2$ and varying EGTA concentrations to yield the Ca^{2+} concentrations specified in the text, as determined using the computer program MaxChelator and the file of constants BERS.CCM (Bers *et al.*, 1994). In assays carried out with anti-PLN mAb, microsomes were incubated with the antibody or control solution for 20 min at 5× their final concentration in the reaction mixture. The final protein concentration of the antibody was 7.5 ng/mL. The remainder of the assay mixture, except the Ca^{2+} buffer, was then added and allowed to temperature-equilibrate for 5 min prior to the addition of Ca^{2+} to start the reaction. When reactions were run at 37 °C, it was necessary to reduce the microsome concentration to 2.5 μg/mL. The filters were washed and counted by liquid scintillation (Antipenko *et al.*, 1997). Changes in calcium uptake and other assays described below are considered statistically significant at a p level of 0.05 or less. Calcium uptake rates, measured as a function of Ca^{2+} concentration, were fit to the Hill equation, $V = V_{max}/[1 + (K_{m(L)}/[L])^N]$, by a nonlinear least-squares procedure, where L represents Ca^{2+} and N is the Hill coefficient.

Ca^{2+}-ATPase Activity. Microsomes (2.4 μg/mL) were incubated in a temperature-equilibrated reaction mixture containing 40 mM histidine hydrochloride, pH 6.8 at either 25° or 37 °C, 120 mM KCl, 5 mM NaN_3, 2 mM $MgCl_2$, 1 mM phospho(enol)pyruvate, 10 units/mL pyruvate kinase, 28.3 units/mL lactic dehydrogenase, 0.2 mM NADH, 0.3 μg/mL A23187, 1 mM ATP, 1 μM okadaic acid, and 160 units/mL PKA catalytic subunit or control solution (see Materials). After 2 min, either 10 mM EGTA or a $CaCl_2$−EGTA buffer solution yielding 0.3 or 9 μM Ca^{2+} was added to start the ATPase reaction. The final concentration of DMSO was 0.9% except when thapsigargin was included in the reaction mixture (see Results), in which case it was 1.8%. The $CaCl_2$−EGTA buffer was prepared as described above except that the radiolabel was omitted. ATPase activity was followed in a spectrophotometer by recording the rate of

decrease in NADH absorbance as described previously (Antipenko *et al.*, 1997). Ca^{2+}-ATPase activity was taken as the difference between ATPase activity measured in the presence and absence of Ca^{2+}. When testing the effect of anti-PLN mAb, the procedure was identical except that anti-PLN mAb, 7.5 ng/mL, or an appropriate control solution was substituted for the PKA catalytic subunit (or control solution).

Steady-State E_2P Formation from $^{32}P_i$. Prior to measurement of steady-state E_2P formation, microsomes (0.36 mg/mL) were incubated under conditions favorable for PLN phosphorylation. The phosphorylation mixture contained (final concentrations) 42 mM histidine hydrochloride, pH 6.8 at 25 °C, 3 mM $MgCl_2$, 1 mM ATP, 1 μM okadaic acid, either 990 units/mL PKA catalytic subunit or control solution (see Materials), and 0.36 mg/mL microsomal protein. After 5 min of incubation, the reaction mixture was chilled on ice and centrifuged in the cold room in an Airfuge centrifuge (Antipenko *et al.*, 1997). The pellets were suspended in a solution containing 5 mM histidine hydrochloride, pH 6.5 at 25 °C, and 1 μM okadaic acid. The protein concentration was determined by the microassay procedure described by Peterson (1977). The phosphorylated and control microsomes (0.25 mg/mL, final concentration) were then added to a temperature-equilibrated reaction mixture containing 40 mM histidine hydrochloride at pH 6.5 at 15, 25, or 37 °C, EGTA (brought to pH 6.5 with Tris at 15, 25, or 37 °C), 5 mM $MgCl_2$, and 1 μM okadaic acid. After a further 1-min equilibration period, 2 mM [^{32}P]phosphoric acid−Tris ($^{32}P_i$), also at pH 6.5 at the three different temperatures, was added to allow steady-state E_2P formation. The specific radioactivity of $^{32}P_i$ was 3.4×10^5 Bq/μmol. After 15 s, acid was added to the reaction tubes and the precipitated protein was washed on the filters as described previously (Antipenko *et al.*, 1997).

E_2P Decomposition. Different preparations of purified microsomes were pooled in order to obtain the required amount of microsomal protein. Prior to measurement of E_2P decomposition, the microsomes were phosphorylated with PKA catalytic subunit or treated under control conditions, as described above, and then were incubated at a concentration of 4 mg/mL in a reaction mixture favorable for [^{32}P]-E_2P formation, also as described above except that the pH of the reaction mixture was 6.0 at 15 °C and 14% (v/v) DMSO was included. One volume of the mixture containing the PKA-phosphorylated or control [$^{32}P_i$]-labeled microsomes was mixed with 16 volumes of a chase solution in a Biologic QFM-5 rapid mixing apparatus (Antipenko *et al.*, 1997). The reactions were quenched at different times ranging from 15 to 200 ms by addition of an equal volume of 10.3% trichloroacetic acid. The acid-quenched samples were applied to filters and processed as described before (Antipenko *et al.*, 1997).

RESULTS

Incubation of purified cardiac microsomes in the presence of increasing concentrations of the catalytic subunit of PKA under conditions favorable for phosphorylation produced a saturable increase in calcium uptake at 9 μM Ca^{2+} of about 39% when 1 μM okadaic acid, a potent phosphoprotein phosphatase inhibitor (MacDougall *et al.*, 1991), was present in the incubation medium (Figure 1). When okadaic acid was absent from the medium, the PKA-induced increase in calcium uptake was only about 31%. Okadaic acid produced a similar increase in calcium uptake across the entire range of PKA concentrations tested, even in the absence of added PKA, suggesting a preexisting equilibrium between intrinsic protein kinase and phosphatase activities. Subsequent calcium uptake and Ca^{2+}-ATPase assays were therefore carried out in the presence of 1 μM okadaic acid and a saturating concentration of 160 units/mL PKA catalytic subunit or control solution. Other preliminary experiments were carried out to establish conditions for identifying an effect of anti-PLN mAb on calcium uptake at 9 μM Ca^{2+}. We observed a reproducible stimulation of calcium uptake that was maximal at an antibody concentration of 7.2 ng/mL upon preincubation of the microsomes at 15, 25, or 37 °C for 20 min (data not shown). These conditions were chosen for further experiments unless otherwise indicated.

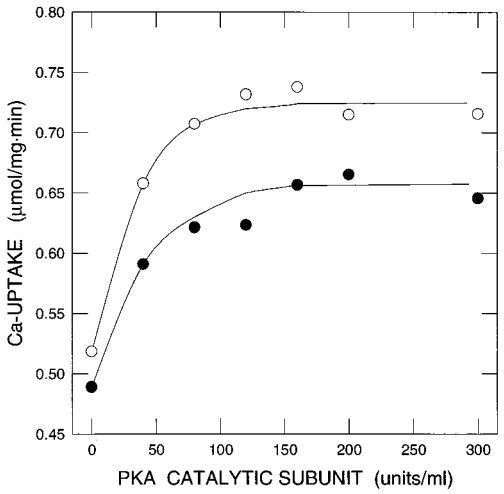

FIGURE 1: Effect of 1 μM okadaic acid on the stimulation of microsomal calcium uptake by PKA catalytic subunit. Purified cardiac microsomes were incubated in the presence (○) and absence (●) of 1 μM okadaic acid and the indicated concentrations of PKA catalytic subunit and assayed for calcium uptake at 9 μM Ca^{2+}.

Ca^{2+} Concentration Dependence of Calcium Uptake. Treatment of microsomes with anti-PLN mAb or PKA catalytic subunit under conditions favorable for phosphorylation produced significant and almost identical increases in calcium uptake over the entire range of Ca^{2+} concentrations tested (Figure 2). When the optimized parameters were obtained by a fit of the data to the Hill equation (see Experimental Procedures), the mean percent change in $V_{max(Ca)}$ of calcium uptake (*i.e.*, a 33% and 37% increase with PKA and anti-PLN mAb, respectively) was found to be similar or somewhat greater than the percent change in $K_{m(Ca)}$ (*i.e.*, a 22% and 31% decrease with PKA and anti-PLN mAb, respectively) (Table 1) and only the change in $V_{max(Ca)}$ was statistically significant at the 0.05 level. There was no significant change in the Hill coefficient.

Calcium uptake rates assayed at 37 °C were up to 2.5-fold those obtained at 25 °C (Figure 3). More importantly, the percent increase following treatment with PKA catalytic subunit was again almost identical to those obtained with anti-PLN mAb over the entire Ca^{2+} concentration range tested (*cf.* Figure 2). At 37 °C, PKA and anti-PLN mAb both increased $V_{max(Ca)}$ by 44% and decreased $K_{m(Ca)}$ by 14% and 11%, respectively (Table 1). Also at 37 °C, the decrease in $K_{m(Ca)}$ observed with PKA and mAb was statistically

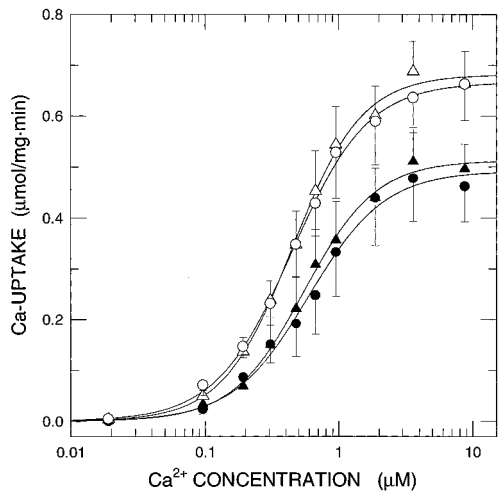

FIGURE 2: Effects of PKA catalytic subunit and anti-PLN mAb on the Ca^{2+} concentration dependence of microsomal calcium uptake assayed at 25 °C. Microsomes were pretreated with either PKA catalytic subunit (○) or PKA control solution (●) or anti-PLN mAb (△) or mAB control solution (▲) and assayed for calcium uptake at the indicated Ca^{2+} concentrations. The symbols represent the means ± SE of three experiments with different microsome preparations. Error bars are omitted where their size is equal to or less than the size of the symbol. The lines are the results of unweighted fits of the data to the Hill equation, as given under Experimental Procedures. The optimized kinetic parameters obtained from these fits are presented in Table 1.

significant in contrast to the findings at 25 °C, where the standard error was considerably larger. As before, there was no change in the Hill coefficient. As an additional control to establish the validity of the use of the control solutions (see Materials) in experiments to test the effects of PKA and anti-PLN mAb, we determined the magnitude of the increase obtained with PKA and mAb using boiled protein preparations as controls. The percent increase with PKA and mAb was 37% ± 9% and 48% ± 8% (mean ± SE, $N = 3$), respectively, which compared favorably with the corresponding percentages shown in Table 1.

Ca^{2+}-ATPase Activity. In order to distinguish whether the changes in calcium uptake associated with microsomal phosphorylation and binding of anti-PLN mAb were effects on the calcium pump protein or on a parallel calcium efflux pathway, we assayed Ca^{2+}-ATPase activity in microsomes treated similarly. A Ca^{2+} ionophore, A23187, was present in the reaction mixture in order to prevent back-inhibition of the Ca^{2+}-ATPase by accumulated calcium in the vesicular lumen. In measurements of calcium uptake, the extent of calcium pump inhibition can be greatly reduced through the use of oxalate, which forms an insoluble precipitate when the intralumenal activities of Ca^{2+} and oxalate reach the solubility product of calcium oxalate. In this case, the rates of calcium uptake are dependent on the oxalate concentration in the medium. In the presence of A23187, in which the membrane is presumably fully permeable to Ca^{2+}, it is not unreasonable to find relatively high Ca^{2+}-ATPase activities compared to the rates of oxalate-facilitated calcium uptake. To confirm that the Ca^{2+}-ATPase activity was attributable to the SR, the Ca^{2+}-ATPase activity was assayed at 37 °C in 9 μM Ca^{2+} in the presence and absence of thapsigargin, a specific inhibitor of sarcoplasmic/endoplasmic reticulum ATPases (Lytton *et al.*, 1991). Thapsigargin (100 nM) reduced the Ca^{2+}-ATPase activity from 1.44 ± 0.19 to 0.02 ± 0.00 μmol/(min·mg) (mean ± SE, $N = 3$) or to 1.6% of the activity observed in its absence.

Ca^{2+}-ATPase activity was assayed under standard conditions at saturating (9 μM) and subsaturating Ca^{2+} concentrations at different temperatures and pH (Table 2). At 0.48 μM Ca^{2+} and 25 °C, the observed 109% increase in Ca^{2+}-ATPase activity with microsomal phosphorylation compares favorably with an 81% increase in calcium uptake, as shown in Figure 2. A 52% increase in Ca^{2+}-ATPase activity observed at the same temperature and 9 μM Ca^{2+} with microsomal phosphorylation was even greater than the increase in $V_{max(Ca)}$ of calcium uptake (assayed under different conditions) shown in Table 1. Also with anti-PLN mAb at 25 °C, the increase in Ca^{2+}-ATPase activity assayed at 9 μM Ca^{2+} (48%) was slightly higher than the increase in $V_{max(Ca)}$ of calcium uptake (37%) shown in Table 1. However, in Ca^{2+}-ATPase assays carried out at 37 °C, the percent increases at 9 μM Ca^{2+} with PKA and anti-PLN mAb (45% and 40%, respectively) were nearly identical to the increase in $V_{max(Ca)}$ observed in the calcium uptake assay (44%, Table 1). Significant increases in Ca-ATPase activity with PKA and anti-PLN mAb were also obtained at pH 7.3, which approximates normal physiological intracellular pH.

Steady-State E_2P Formation from P_i and E_2P Decomposition. Steady-state E_2P formation was assayed in order to assess whether the observed increase in $V_{max(Ca)}$ with microsomal phosphorylation or anti-PLN mAb may, at least in part, be attributable to an increased rate of E_2P decomposition, as previously demonstrated in trypsin-treated microsomes (Antipenko *et al.*, 1997). Steady-state E_2P formation from P_i represents an equilibrium between E_2P formation and decomposition. An acceleration in E_2P decomposition will be reflected in a decreased steady-state E_2P level provided no equivalent increase occurs in the rate of E_2P formation. We measured steady-state E_2P formation at three different temperatures in order to determine its temperature dependence, particularly since we were able to run our kinetic measurements of E_2P decomposition only at 15 °C due to technical limitations (see below).

Steady-state E_2P formation decreased by 36% at 15 °C and by 29% at 25 °C, but was unchanged at 37 °C as a result of treatment of microsomes with PKA catalytic subunit (Table 3). There was, moreover, a decrease in steady-state E_2P formation with increasing temperature in control microsomes and an increase in steady-state E_2P formation with temperature in PKA-treated microsomes. This suggests an increased rate of E_2P decomposition with temperature in the control microsomes and a decreased effectiveness of PKA. Kinetic measurement of E_2P decomposition at 15 °C shows that PKA treatment does indeed increase the rate of E_2P decomposition, in this case by 70% (Figure 4). Similar measurements were attempted using anti-PLN mAb. However under the conditions tested, we observed only a slight decrease in E_2P formation (about 10−20%), which is likely to be due to a subsaturating, cost-limited amount of mAb used relative to the amount of microsomal protein required to obtain enough counts on the filter.

DISCUSSION

The present data demonstrate that PKA-catalyzed cardiac microsomal phosphorylation and binding of anti-PLN mAb both produce significant and similar increases in $V_{max(Ca)}$ of

Table 1: Kinetic Parameters for Calcium Uptake by Purified Cardiac Microsomes Treated with PKA or Anti-PLN Monoclonal Antibody[a]

treatment	$V_{max(Ca)}$ [μmol/(mg·min)]	% change	$K_{m(Ca)}$ (μM)	% change	ΔpCa	Hill coefficient *N*
			Temperature: 25 °C			
control	0.51 ± 0.05^{a}		0.59 ± 0.14^{e}			1.68 ± 0.12
PKA	0.68 ± 0.06^{a}	+33	0.46 ± 0.06^{e}	−22	0.11	1.62 ± 0.06
control	0.49 ± 0.08^{b}		0.70 ± 0.18^{f}			1.59 ± 0.16
anti-PLN mAb	0.67 ± 0.07^{b}	+37	0.48 ± 0.04^{f}	−31	0.18	1.52 ± 0.12
			Temperature: 37 °C			
control	1.17 ± 0.10^{c}		0.49 ± 0.02^{g}			1.61 ± 0.09
PKA	1.69 ± 0.10^{c}	+44	0.42 ± 0.02^{g}	−14	0.07	1.63 ± 0.07
control	1.13 ± 0.07^{d}		0.55 ± 0.03^{h}			1.64 ± 0.05
anti-PLN mAb	1.63 ± 0.05^{d}	+44	0.49 ± 0.05^{h}	−11	0.15	1.56 ± 0.08

[a] Microsomes were preincubated either in the presence and absence (control) of the catalytic subunit of PKA under conditions favorable for phosphorylation or in the presence and absence (control) of anti-PLN mAb and assayed for calcium uptake at the indicated temperature. The values are the means ± SE of the optimized parameters that were obtained in separate unweighted fits to the Hill equation of the three data sets shown in each of Figures 2 and 3. The differences in values identified by the same superscript are significant at $p < 0.05$ when tested by Student's *t*-test for paired (*a, b, g*), unpaired (*h*), or both paired and unpaired (*c, d*) variates or are not significant (*e, f*). See Experimental Procedures for additional details.

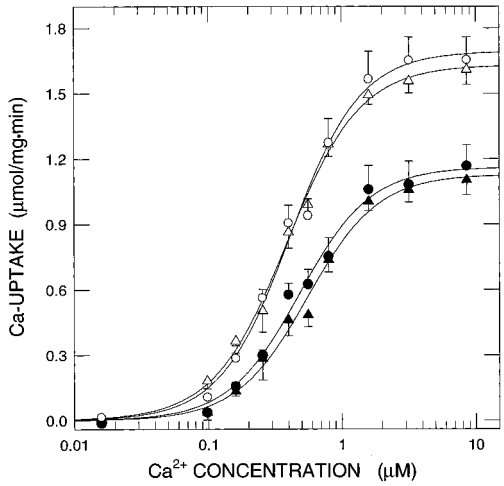

FIGURE 3: Effects of PKA catalytic subunit and anti-PLN mAb on the Ca^{2+} concentration dependence of microsomal calcium uptake assayed at 37 °C. The experiments were carried out as described in the legend to Figure 2 except for the change in temperature. The symbols are the same as in Figure 2.

calcium uptake and decreases in $K_{m(Ca)}$ (Figures 2 and 3, Table 1). The fact that comparable results were obtained when Ca^{2+}-ATPase activity was assayed (Table 2) indicates a modulation of the calcium pump protein rather than activation of some parallel Ca^{2+} efflux pathway. The following factors appear to contribute to our ability to demonstrate these findings: (i) the use of purified cardiac microsomes; (ii) the presence of 1 μM okadaic acid in assay buffers involving phosphorylation reactions; (iii) the use of appropriate controls for test substances; and (iv) proven linearity of each reaction with time.

All of the previously cited laboratories studying regulation of the SR calcium pump by PLN in cardiac microsomes have utilized crude microsome preparations. The purified cardiac microsomes used in the present study are enriched in SR membranes that are devoid of detectable ruthenium-sensitive calcium release channels (see introduction and Experimental Procedures) and are less likely than crude microsomes to contain contaminants released from other subcellular compartments that may interact with the calcium pump and its regulation by PLN. The calcium pump−PLN system has previously been shown to be sensitive to a wide variety of substances that are polyanionic, polycationic (Xu & Kirchberger, 1989), or amphiphilic (Antipenko *et al.*, 1997) in nature. These considerations apply also to cardiac homogenates (Luo *et al.*, 1994) or crude microsomes prepared from cultured cells (Odermatt *et al.*, 1996). The reconstitution of purified Ca^{2+}-ATPase and PLN into lipid vesicles of known phospholipid composition avoids these problems but raises other issues: Critical components like phosphatidylinositol phosphates, which have been reported to accelerate E_2P decomposition (Starling *et al.*, 1995*b*), may be lacking. Also, despite the treatment of the reconstituted vesicles with Bio-Beads to remove nonionic detergents (Reddy *et al.*, 1995), the possibility that low levels of residual detergent remain present has not been eliminated. We have previously demonstrated that low concentrations of commonly used nonionic detergents (below the critical micelle concentration) interfere specifically with the regulation of $V_{max(Ca)}$ by PLN in cardiac microsomes but have no apparent effect on calcium uptake by fast skeletal muscle microsomes (Lu & Kirchberger, 1994). Finally, the orientation of the proteins incorporated into the artificial membrane bilayer may differ from the orientation in the native membrane (Reddy *et al.*, 1995).

Cardiac microsome preparations have previously been shown to contain phosphoprotein phosphatase 1 activity capable of dephosphorylating PLN (Iyer *et al.*, 1988; Macdougall *et al.*, 1991). It is therefore not surprising to find a significant increase in the stimulation of calcium transport by PKA catalytic subunit in the presence of 1 μM okadaic acid (Figure 1). While thus far not generally used in studies with microsome preparations (*e.g.*, Odermatt *et al.*, 1996; Reddy *et al.*, 1996), okadaic acid has been shown to increase PLN phosphorylation and the force of contraction in cardiomyocytes (Neuman *et al.*, 1993).

Regarding other factors that may affect the demonstration of PLN's regulation of $V_{max(Ca)}$, the importance of appropriate controls when testing the effects of PKA catalytic subunit and other substances has been stressed previously (Odermatt *et al.*, 1996). In the present study, nonlinearity of the calcium uptake reaction or Ca^{2+}-ATPase activity was avoided by reducing either incubation time or microsomal protein concentration or both. Deviation from linearity is more likely

Table 2: Effect of PKA and Anti-PLN Monoclonal Antibody on Cardiac Microsomal Ca^{2+}-ATPase Activity Assayed at Different Temperatures and pH[a]

		25 °C				37 °C			
		0.48 μM Ca^{2+}		9 μM Ca^{2+}		0.40 μM Ca^{2+}		9 μM Ca^{2+}	
treatment	pH	activity [μmol/(mg·min)]	% increase	activity [μmol/(mg·min)]	% increase	activity [μmol/(mg·min)]	% increase	activity [μmol/(mg·min)]	% increase
control	6.8	0.11 ± 0.01		0.48 ± 0.03		0.30 ± 0.04		1.72 ± 0.18	
PKA		0.23 ± 0.01	109	0.73 ± 0.07	52	0.68 ± 0.10	127	2.49 ± 0.30	45
control	7.3							1.25 ± 0.08	
PKA								1.62 ± 0.14	30
control	7.3							1.36 ± 0.52	
anti-PL mAb								1.90 ± 0.08	40
control	6.8			0.46 ± 0.04				1.47 ± 0.17	
anti-PL mAb				0.68 ± 0.05	48			2.06 ± 0.26	40

[a] The microsomes were pretreated with either the catalytic subunit of PKA or a control solution under conditions favorable for phosphorylation or with anti-PLN mAb or control solution and then assayed for Ca^{2+}-ATPase activity at the indicated temperature and pH. The values are the means ± SE of three experiments carried out with different microsome preparations. All increases were significant at $p < 0.05$ when tested by Student's *t*-tests for paired or unpaired variates. See Experimental Procedures for additional detail.

Table 3: Effect of Protein Kinase A- (PKA-) Catalyzed Microsomal Phosphorylation on Steady-State E_2P Formation from P_i at Different Temperatures[a]

	15 °C		25 °C		37 °C	
treatment	nmol of E_2P/mg	%	nmol of E_2P/mg	%	nmol of E_2P/mg	%
control	1.82 ± 0.1[a]	100	1.66 ± 0.02[b,c]	100	1.56 ± 0.01[c]	100
PKA	1.16 ± 0.14[a,d]	64	1.18 ± 0.02[b,e]	71	1.63 ± 0.09[d,e]	104

[a] Shown are the means ± SE obtained in three experiments with different microsome preparations. Microsomes were treated with the catalytic subunit of PKA or control solution, centrifuged, and resuspended in buffer prior to measurement of steady-state E_2P formation from P_i. Values identified by the same superscript are significant at $p < 0.05$ when tested by Student's *t*-test for unpaired (*a, d*) or both paired and unpaired (*b, c, e*) variates.

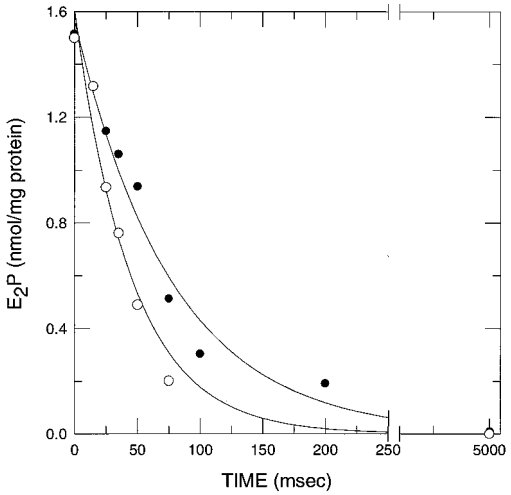

FIGURE 4: Effect of microsomal phosphorylation by PKA catalytic subunit on E_2P decomposition. Phosphorylated and control microsomes were incubated in the presence of $^{32}P_i$ to form E_2P and then mixed at 15 °C in a QFM-5 rapid mixing system with 16 volumes of a chase solution. At the indicated times the reaction was quenched with an equal volume of 10.3% trichloroacetic acid, as described in Experimental Procedures. In order to obtain the 5 s data points, the reactants were mixed manually. To obtain the zero-time points, the quench solution was added manually to the ^{32}P-labeled microsomes. Each data point represents an average of two determinations. The lines represent single-exponential fits with rate constants of 12.9 (●) and 22.0 (○) s^{-1}.

to occur at high levels of calcium pump activity and may result in falsely low rates.

The 0.07−0.18 pCa unit shift produced by PKA catalytic subunit-catalyzed microsomal phosphorylation or anti-PLN mAb (Table 1) is significantly less than the 0.3−0.5 pCa unit shift generally attributed to PLN in crude microsomes. A relatively small effect of PLN on calcium pump properties in studies with artificial systems was noted before (Reddy *et al.*, 1995) but was attributed to technical difficulties. In a subsequently reported reconstitution system (Hughes *et al.*, 1996), the shift in $K_{m(Ca)}$ was closer to the 0.3−0.5 pCa unit shift generally attributed to PLN in crude microsomes. A possible factor in the large variation in the shifts reported in pCa is presumably a lack of accurate values for $V_{max(Ca)}$ in reconstituted systems and crude microsome preparations resulting in inaccurate values for $K_{m(Ca)}$.

Besides the demonstration of an increase in $V_{max(Ca)}$ of the cardiac SR calcium pump upon treatment of microsomes with PKA or anti-PLN mAb, a second significant finding in the present study is the increased rate of E_2P decomposition as a result of PKA catalytic subunit-catalyzed microsomal phosphorylation (Figure 4) and the related decrease in steady-state E_2P formation from P_i (Table 3). Our findings fail to support the conclusion of Cantilina *et al.* (1993) that PLN regulates a single kinetic step in the catalytic cycle of the Ca^{2+}-ATPase, namely, the conformational change in the calcium pump protein associated with the binding of the first of two Ca^{2+}; this effect is overcome at saturating Ca^{2+} concentrations. No effect attributable to PLN was observed when measuring steady-state Ca^{2+} binding to the calcium pump protein or the rate of breakdown of phosphoenzyme formed in the presence of [^{32}P]ATP upon addition of a chase solution containing nonradiolabeled ATP and Ca^{2+} to the reaction mixture. Our previous study with trypsin-treated (purified) microsomes (Antipenko *et al.*, 1997) provided the initial direct demonstration that the rate of E_2P decomposition is modulated by PLN, in addition to its effect on Ca^{2+} binding. A change in steady-state E_2P formation from P_i upon binding of anti-PLN mAb was already demonstrated by Shi *et al.* (1996), although in the present study only a minor change was observed, probably as a result of a subsaturating concentration of antibody. The latter reason could explain also the lack of effect of anti-PLN mAb on the decay of ^{32}P-labeled phosphoenzyme formed from ^{32}P-

ATP upon addition of a chase solution containing unlabeled ATP (Cantilina *et al.*, 1993).

Our finding of a PLN-mediated change in E_2P decomposition supports that of Nediani *et al.* (1996), who observed a stimulation of calcium pump activity in the presence of a purified cytoplasmic acylphosphatase. In the latter study, the stimulation of calcium uptake produced by the purified acylphosphatase was decreased upon microsomal phosphorylation, which was attributed to a PLN phosphorylation-induced increase in the rate of decomposition of the acylphosphoprotein intermediate(s) of the Ca^{2+}-ATPase (primarily E_2P), hence reducing the increase in dephosphorylation attributable to the purified acylphosphatase. Although a PLN-mediated change in E_2P decomposition could account for or at least contribute to the increased $V_{max(Ca)}$ of calcium uptake seen at 25 °C upon microsomal treatment with PKA, it is unlikely to account for such an increase at 37 °C since no difference in steady-state E_2P formation was observed at this higher temperature (Table 3). However, an inhibition by PLN of the calcium transport step ($E_1PCa_2 \rightarrow E_2PCa_2$), as was reported by Hughes *et al.* (1994), might become evident at the higher temperature. Conceivably, PLN could inhibit several steps in the catalytic cycle of the Ca^{2+}-ATPase but its inhibitory effect on any one step becomes evident only when other factors such as temperature, pH, or ionic strength render that particular step rate-limiting for the overall catalytic cycle. Thus at 37 °C, E_2P decomposition, which in fast skeletal SR is highly sensitive to temperature (Champeil *et al.*, 1986; Lacapère *et al.*, 1981), may be accelerated by temperature alone so that in measurements of steady-state E_2P formation in cardiac microsomes, the level is significantly reduced as the temperature is increased from 15 to 37 °C (Table 3). PLN phosphorylation also accelerates E_2P decomposition (Figure 4) but, it may be speculated, the phosphorylation-dependent acceleration might vary inversely with temperature so that steady-state E_2P formation increases from 15 to 37 °C, where it is approximately equal in control and phosphorylated microsomes. The observed increase in steady-state E_2P levels with temperature in phosphorylated microsomes (Table 3) might also be related to a disproportionately greater acceleration of E_2P formation than E_2P decomposition.

Although much work has been devoted to characterizing the biochemical effects of PLN on Ca^{2+}-ATPase, the physical basis for the regulation of the cardiac SR calcium pump by PLN has been studied to a far lesser extent. A biophysical model for the mechanism of the regulation has been proposed by Thomas and co-workers on the basis of electron paramagnetic resonance and time-resolved anisotropy measurements (*e.g.*, Shi *et al.*, 1996). According to this model, unphosphorylated PLN produces aggregation of the Ca^{2+}-ATPase into large oligomers within which the pump expresses reduced activity, whereas PLN phosphorylation causes dissociation of these aggregates into dimers or monomers of Ca^{2+}-ATPase with a concomitant increase in activity. However, no evidence for a PLN-mediated aggregation−deaggregation of calcium pump units was observed by Negash *et al.* (1996) on the basis of cross-linking experiments or by Starling *et al.* (1995a) from electron paramagnetic resonance measurements of spin-labeled ATPase. Nevertheless, despite the controversial nature of the physical basis for the regulation of the SR calcium pump by PLN, the presently described kinetic effects of PLN, obtained with purified microsomes, reflect the overall effect of PLN on transmembrane calcium fluxes.

The present data demonstrate that the cardiac SR has at least the potential of regulating its calcium pump through PLN phosphorylation/dephosphorylation over a wide range of Ca^{2+} concentrations. Typical previously reported values of $K_{m(Ca)}$ for cardiac microsomes are around 0.7 μM at 25 °C (*e.g.*, Odermatt *et al.*, 1996). If peak systolic Ca^{2+} concentrations are between 0.8 and 1 μM, as estimated by Peeters *et al.* (1987), then an ability of PLN to regulate only the $K_{m(Ca)}$ of the Ca pump might account for the abbreviation of systole seen in response to β-adrenergic agonists in the intact heart. However, the peak systolic Ca^{2+} concentrations reported by Peeters *et al.* (1987) may be underestimated compared to the value predicted by Fabiato *et al.* (1981) of greater than 1 μM. Moreover, systolic Ca^{2+} concentrations in the β-adrenergically stimulated heart are likely to be increased above the estimated basal values, whereas the $K_{m(Ca)}$ of the calcium pump is decreased, typically, in *in vitro* studies, to 0.4−0.5 μM (*e.g.*, Odermatt *et al.*, 1996; Table 1). Also, contrary to previous reports in which a decrease in $K_{m(Ca)}$ of approximately 50% is not uncommon (*e.g.*, Odermatt *et al.*, 1996), in the present study (Table 1) the $K_{m(Ca)}$ was decreased by less than 15% by PLN phosphorylation or anti-PLN mAb when calcium uptake was assayed at 37 °C. It could thus be argued that under such conditions [*i.e.*, increased peak systolic Ca^{2+} concentrations with catecholamines with relatively little effect of PLN phosphorylation on $K_{m(Ca)}$], an increase in $V_{max(Ca)}$ of the SR calcium pump upon PLN phosphorylation would play an important role in mediating the β-agonist-induced abbreviation of systole and decreased time to peak tension. In view of these and other considerations, a conclusion as to the relative importance of the presently demonstrated effects of PLN phosphorylation on both $K_{m(Ca)}$ and $V_{max(Ca)}$ of microsomal calcium uptake in the intact heart must await further study.

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BI971109V