Comparison of the Effects of Phospholamban and Jasmonate on the Calcium Pump of Cardiac Sarcoplasmic Reticulum

EVIDENCE FOR MODULATION BY PHOSPHOLAMBAN OF BOTH Ca\(^{2+}\) AFFINITY AND \(V_{\text{max}}(\text{Ca})\) OF CALCIUM TRANSPORT*

(Received for publication, May 6, 1996, and in revised form, November 7, 1996)

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Regulation of the calcium pump of the cardiac sarcoplasmic reticulum by phosphorylation/dephosphorylation of phospholamban is central to the inotropic and lusitropic effects of \(\beta\)-adrenergic agonists on the heart. In order to study the mechanism of this regulation, we first obtained purified ruthenium red-insensitive microsomes enriched in sarcoplasmic reticulum membranes. The kinetics of microsomal \(\text{Ca}^{2+}\) uptake after phosphorylation or trypsin treatment, which cleaves the inhibitory cytoplasmic domain of phospholamban, were then compared with those in the presence of jasmonate, whose effects on the kinetics of fast skeletal muscle \(\text{Ca}^{2+}\)-ATPase are largely known. All three treatments increased \(V_{\text{max}}(\text{Ca})\) at 25 °C and millimolar ATP; phosphorylation and trypsin decreased the \(K_m(\text{Ca})\) while jasmonate increased it. Trypsin and jasmonate increased the rate of \(E_{\text{2P}}\) decomposition 1.8- and 3.0-fold, respectively. The effects of phospholamban phosphorylation and jasmonate on the \(\text{Ca}^{2+}\)-ATPase activity paralleled their effects on \(\text{Ca}^{2+}\) uptake. Our data demonstrate that phospholamban regulates \(E_{\text{2P}}\) decomposition in addition to the known increase in the rate of a conformational change in the \(\text{Ca}^{2+}\)-ATPase upon binding the first of two \(\text{Ca}^{2+}\). These steps in the catalytic cycle of the \(\text{Ca}^{2+}\)-ATPase may contribute to or account for phospholamban’s effects on both \(V_{\text{max}}(\text{Ca})\) and \(K_m(\text{Ca})\). Whose relative magnitude may vary under different experimental and, presumably, physiological conditions.

Sequestration of \(\text{Ca}^{2+}\) by the calcium pump of SR membranes accounts for the major portion of the \(\text{Ca}^{2+}\) removed from the cytoplasm during cardiac muscle relaxation (1). Hence, factors that affect SR calcium pump activity are expected to have marked effects on the mechanical properties of the heart. This has been found to be the case with PLN, the natural protein regulator of the cardiac SR calcium pump (2). PKA-catalyzed phosphorylation of PLN, which leads to increased calcium pump activity, plays a central role in both the lusitropic and inotropic effects of catecholamines on the myocardium. Evidence for this dual role of PLN has been obtained in experiments with cardiomyocytes (3) and with PLN gene-deficient mice, whose hearts exhibited mechanical properties virtually identical to fully catecholamine-stimulated hearts from wild-type mice (4).

Despite major progress in our understanding of the physiological role of PLN, the molecular mechanism of its regulation of the SR calcium pump remains poorly understood. Unphosphorylated PLN is believed to function as a calcium pump inhibitor that becomes inactive upon phosphorylation by PKA (5–7). Removal of this inhibitory influence is also produced in vitro by proteolytic cleavage of the cytoplasmic domain of PLN (6) or by incubation of SR membranes in the presence of certain polyanions (8) or monoclonal antibodies against PLN (7).

Almost all of the laboratories that have studied the effect of PKA-catalyzed phosphorylation of PLN or trypsin treatment of cardiac microsomes on the kinetics of calcium uptake or \(\text{Ca}^{2+}\)-ATPase activity have reported a phosphorylation-induced increase in the apparent equilibrium constant for \(\text{Ca}^{2+}\) binding, \(K_m(\text{Ca})\) (9–17). Some investigators have concluded that this is the only effect of PLN phosphorylation by PKA (4, 11–16), while others have reported that \(V_{\text{max}}(\text{Ca})\) is also increased (2, 9). Another laboratory reported that phosphorylation affects \(V_{\text{max}}(\text{Ca})\) but not \(K_m(\text{Ca})\) (18). In our studies (9, 10), we interpreted the actions of PLN in terms of effects on different steps in the catalytic cycle of the \(\text{Ca}^{2+}\)-ATPase, which may become rate-limiting under different experimental conditions, although rates of individual steps in the catalytic cycle were not measured.

The major elementary steps in the catalytic cycle of the cardiac \(\text{Ca}^{2+}\)-ATPase are summarized in Scheme 1, which represents a modification of the models given in Refs. 19 and 20. The enzyme is believed to exist in two major functional states, \(E_1\) and \(E_2\), whose equilibrium is shifted toward \(E_1\) in the presence of either ATP or \(\text{Ca}^{2+}\). ATP, in the presence of \(\text{Mg}^{2+}\), binds to \(E_1\) with high affinity and accelerates the conversion of \(E_2\) to \(E_1\) and the conformational change involved in the binding of \(\text{Ca}^{2+}\), which also binds to \(E_1\) with high affinity (step 2) (19, 21, 22). ATP may also bind to \(E_2\) with reduced affinity (step 1a) and accelerate the conversion of \(E_2\) to \(E_1\), followed by high affinity binding of \(\text{Ca}^{2+}\) to the latter (23) (step 2a). After hydrolysis of the ATP and occlusion of \(\text{Ca}^{2+}\) (step 3), ADP is released on the cytoplasmic side of the SR membrane (step 4), and \(\text{Ca}^{2+}\) is vectorially transferred across the membrane (step 5) and released on its luminal side (step 6). Finally, \(E_{\text{2P}}\) decomposition and release of inorganic phosphate on the cytoplas-
mic side of the membrane return the enzyme to the ligand-free $E_2$ state (step 7). However, ATP (shown in parentheses) may bind with reduced affinity to the enzyme already at steps 3, 5, and 7 of the cycle and produce forward acceleration of these transitions (20, 22).

In the present study, we compare the effects of PLN phosphorylation or trypsin treatment of purified cardiac microsomes on some of the properties of the calcium pump of SR membranes with those produced by the addition of jasmonate. Jasmonate, a minor component of peppermint oil, has been shown to accelerate $E_{2P}$ decomposition (step 7) in fast skeletal muscle SR, causing a decrease in the equilibrium constant for phosphorylation of $E_2$ by $P_i$ (step 7) (24). This effect was attributed to a specific interaction of jasmonate with the fast skeletal muscle Ca$^{2+}$-ATPase rather than to general perturbation of the phospholipid bilayer of the SR membrane, although an interaction with the Ca$^{2+}$-ATPase that involves its relationship to phospholipids cannot be ruled out. Using our newly developed preparation of ruthenium red-insensitive cardiac microsomes enriched in SR membranes, we demonstrate an increase in the rate of $E_{2P}$ decomposition of the Ca$^{2+}$-ATPase as a result of treatment of these membranes with jasmonate or trypsin. This increase may account for or contribute to an observed increase in the $V_{\text{max}}$ (Ca$^2+$) of calcium uptake that is observed with similar treatment or as a result of PKA-catalyzed phosphorylation of the microsomes.

**EXPERIMENTAL PROCEDURES**

**Materials**—Cis-jasmonate was purchased from Aldrich. PKA was partially purified from bovine heart (25). Utrapure sucrose was obtained from Schwarz/Mann Biotec, and ruthenium red, the catalytic subunit of bovine heart PKA, and A23187 were from Sigma. $^{32}$P and $[\beta-\gamma^32P]ATP$ were obtained from DuPont NEN. Unless otherwise stated, the PKA used was a preparation that we partially purified from bovine heart and dialyzed against 5 mmol/l histidine-HCl, pH 6.8 (25); the final solution against which the protein kinase was dialyzed was used as a control solution. When the catalytic subunit of PKA was used, a control solution containing all of the salts and other reagents present in the solution in which the enzyme was dissolved was prepared. Enzymes and phospho(enol)pyruvate for the ATPase assay were obtained from Boehringer Mannheim. Cellulose polyethyleneimine-coated plastic thin layer sheets were obtained from J. T. Baker. All other reagents were obtained as previously reported (9, 10).

**Preparation of Microsomes**—Crude canine cardiac and fast skeletal muscle microsomes, derived from vastus lateralis muscle of male New Zealand White rabbits, were prepared as described previously (6). The muscle microsomes, derived from vastus lateralis muscle of male New Zealand White rabbits, were prepared as described previously (6). The preparations of the crude microsomes were suspended with the aid of a Dounce homogenizer in two 6-ml aliquots of buffer G containing 18 mm PIPES-KOH, pH 6.8, and 120 mm KCl, each to a protein concentration not greater than 50 mg/ml. A sucrose step gradient was prepared by loading two or more Beckman SW28 centrifuge tubes as follows: 5 ml of 40% (w/w), 8 ml of 33%, 10 ml of 30%, and 7 ml of 25% sucrose in buffer G. All procedures were carried out in the cold room or in ice except that the concentration of each sucrose solution was checked at 23°C with a refractometer and adjusted prior to chilling. Centrifugation was carried out at 20,000 rpm (70,000 × g$_{\text{rot}}$) for 20 h. The fractions at the density interfaces were collected with the aid of a 5-ml glass syringe and designated $F_1$ (topmost), $F_2$, and $F_3$. The sucrose concentration of each of the fractions was adjusted to 12% by the addition of buffer G. Following centrifugation of the fractions in a Beckman Ti50 rotor at 29,000 rpm (101,600 × g$_{\text{rot}}$) for 90 min, the pellets were suspended in buffer C (10 mM Hepes, 10 mM MgCl$_2$, 0.1 mM dithiothreitol) at final concentrations ranging from 6.1 to 15.0 mg/ml and stored in liquid nitrogen. Microsomal protein concentrations were determined using the biuret procedure with bovine serum albumin as the standard. The yields of crude and purified cardiac microsomes were approximately 0.9 and 0.1 mg of protein, respectively per gram, wet weight, of left ventricle. The sensitivity of microsomal calcium uptake to 5 μM ruthenium red, an inhibitor of the ligand-gated Ca$^{2+}$ release channel (26), was used as a functional criterion for the absence of these channels. No difference in calcium uptake rates was found in purified microsomes when the assays were run under standard conditions (at 25°C) at 11 μM Ca$^{2+}$, as described below, or at 37°C in the presence of 5 mM oxalate. Ruthenium red was tested at concentrations ranging from 0.5 to 50 μM. Concentrations higher than 5 μM produced no further stimulation of calcium uptake in microsomal fractions containing Ca$^{2+}$ release channels, and concentrations higher than 25 μM produced inhibition.

**Calcium Uptake**—Oxalate-facilitated calcium uptake by microsomes was measured by a filtration procedure described previously (9). In some experiments the microsomes were pretreated with trypsin at a maximum stimulatory concentration of 0.01 μg/ml, inhibitor-inactivated trypsin as a control (9). After the addition of 0.12 mg/ml trypsin inhibitor to the trypsin-treated microsomes, both reaction tubes were kept on ice as aliquots were being removed for assay. The composition of the standard assay mixture was 40 mM histidine-HCl, pH 6.8, at 25°C, 120 mM KCl, 5 mM Na$_2$ATP, 4 mM phosphoenolpyruvate, 0.2 mg/ml pyruvate kinase, 2 mM MgCl$_2$, 2.5 mM oxalate-Tris, 1 mM ATP, 0.01 mg/ml microsomes, and a 250 μM-CaCl$_2$-EGTA buffer consisting of 125 μM CaCl$_2$ and different concentrations of EGTA to yield the Ca$^{2+}$ concentrations specified below. Ca$^{2+}$, Mg$^{2+}$, and Mg-ATP concentrations were calculated as described previously (27) except that a Ca-EGTA binding constant of 10$^6$ M$^{-1}$ was used. The specific radioactivity of the $^45$Ca was about 1.3 × 10$^6$ Bq/μmol at 11 μM Ca$^{2+}$. When calcium uptake was assayed over a range of Ca$^{2+}$ concentrations extending from 0.02 to 11 μM, the specific radioactivity was progressively decreased from 1.3 × 10$^6$ Bq/mmol calcium. After 2 and 4 min of incubation, aliquots of the reaction mixture were filtered and processed as described previously (9). Under some conditions producing high calcium uptake rates, samples were removed after 1 and 2 min. Zero time samples were obtained from reaction mixtures from which ATP was omitted. Also, in some experiments, reactions were run at 37°C. The pH of all assay solutions used in this study was adjusted for the temperature at which the assay was run. Under all conditions tested, calcium uptake was linear with time and protein concentration. The optimized kinetic parameters for calcium uptake, measured as a function of either Ca$^{2+}$ or Mg-ATP concentration, were fit to the Hill equation, $V = V_{\text{max}}/(1 + (K_{a_1} + (K_{a_2} + (K_{a_3} + (K_{a_4} + (K_{a_5} + (1) + (K_{a_6} + (K_{a_7} + (K_{a_8} + (K_{a_9} + (1) + (1))))))))$. The Hill coefficients were calculated from the Hill equation.

Measurement of calcium uptake by phosphorylated or control microsomes was carried out as follows. The microsomes (13 μg of protein/ml) were incubated at 25°C in the standard reaction mixture except that the 45CaCl$_2$-EGTA buffer was omitted and the reaction mixture was present at a 1.35-fold higher concentration and contained 2.7 μM cyclic AMP and either PKA at a concentration of 0.30 or 1.0 μg/ml or a control solution (see “Materials”). After a 2-min incubation, an equivalent volume of either distilled water or jasmonate was added to a concentration, at this point, of 1.1 mM, and the incubation was continued for an additional 2 min prior to a final addition of Ca$^{2+}$ and to start the calcium uptake reaction. The addition of the Ca$^{2+}$ buffer diluted the jasmonate solution to 1.0 mM, while both additions reduced the concentrations of the remaining reagents in the calcium uptake reaction mixture to those specified above for the standard assay medium; the final concentrations of cyclic AMP and PKA were 2 μM and either 0.30 or 0.75 μg of protein/ml, respectively. Jasmonate was added subsequent to the phosphorylation reaction to avoid potential effects of jasmonate on this reaction. Previous studies have shown that PLN is the major protein phosphorylated by PKA in crude cardiac microsomes (28), and it was estimated to account for greater than 95% of the phosphate incorporation in our purified microsomes.

When the catalytic subunit of PKA was used instead of the holoenzyme, microsomes were incubated in the standard reaction mixture.
described above but lacking the CaCl2-EGTA buffer and including either 150 units/ml of catalytic subunit or an equivalent volume of a control solution (see "Materials"). After incubating the microsomes together with the PKA for 2 min, 2.5 mM oxalate was added to start the calcium uptake reaction.

Ca2+-ATPase Activity—Ca2+-ATPase activity was measured at either 25 or 37°C by following the rate of decrease in NADH absorbance at 340 nm in an enzyme-linked assay according to Norby (29) with the following modifications. The reaction mixture contained 40 mM histidine-HCl, pH 6.8; 120 mM KCl; 5 mM Na2Ni; 2 mM MgSO4; 1 mM ATP; 1 mM phosphoenolpyruvate; 10 units/ml pyruvate kinase; 28 units/ml lactate dehydrogenase; 0.2 mM NADH; either a CaCl2-EGTA buffer system, as described above, to yield either 0.32 or 11 mM Ca2+ or 10 mM EGTA; either 0.11 mg/ml PKA and 2 μM cAMP or control solution (see above); 2.4 μg of microsomal protein/ml; and either 1 mM jasmonol or an equivalent volume of distilled water. Prior to its addition to the reaction mixture, the A23187 was dissolved in Me2SO, which was present at a final concentration of 0.3%. A blank was run with the same reaction mixture except that, instead of microsomes, an equivalent volume of buffer B was present; the blank value was subtracted from all values obtained with microsomes. Rates of ATPase activity were obtained using the Kinetic program in a Shimadzu UV 160U spectrophotometer. The cuvette containing the reaction mixture without the microsomes, jasmonol or control solution, and Ca2+ or EGTA was placed in the spectrophotometer, preset at either 25 or 37°C, and after 2 min, the microsomal aliquot was added to buffer B. After an additional 2 min to allow for the phosphorylation or control reaction, jasmonol was added, followed by the addition of Ca2+ or EGTA 1 min later, when the Kinetic program was started. The reaction was allowed to proceed for 6 min, which was the length of time necessary for complete temperature equilibration after the various additions to the cuvette, as determined from a negligibly slower rate than during the next 3 min, which was the interval used to obtain the ATPase activity. During this time, the reactions were linear with respect to time and microsomal protein concentration.

Steady-state E2P Formation from 32P—Prior to measurement of E2P formation, microsomes (1.5 mg/ml) that had previously been suspended in KCl-free buffer B were incubated at 25°C for 2 min in 40 mM histidine-HCl, pH 6.8, and 0.01 mg/ml trypsin in the presence (control) and absence of 0.12 mg/ml trypsin inhibitor. After the addition of trypsin inhibitor to the trypsin-treated microsomes, both reaction tubes were kept on ice and used directly for the assay of steady-state E2P formation. Our assay was based largely on the findings of Starling et al. (24) with skeletal muscle SR. The microsomes (0.21 mg/ml) were incubated at 37°C in our standard reaction mixture for measuring E2P formation, consisting of 50 mM MES-Tris, 5 mM MgCl2, 5 mM Na2Ni, 10 mM EGTA-Tris, and 2.0 mM phosphoric acid-Tris (32Pi) at a final pH of 6.8. The specific radioactivity of 32Pi was 3.4 × 104 cpm/μmol. Assays were also carried out at incubation temperatures of 15 and 37°C. The microsomes were added to the temperature-equilibrated reaction mixture lacking 32Pi, and incubated for 2 min, after which the phosphorylation reaction was started by the addition of 32Pi. Reaction stopped after 15 s by the addition of 7 volumes of an ice-cold solution containing 1 M perchloric acid, 100 mM orthophosphate, and 20 mM pyrophosphate. The acid-quenched reaction mixtures were each visualized under UV light, as well as appropriate blanks, were collected and counted in separate scintillation vials. Results were expressed as the percentage of the total counts found in the spot corresponding to ATP.

RESULTS

Although jasmonol has been reported to increase Ca2+-ATPase activity in fast skeletal muscle SR as a result of an interaction with the calcium pump protein, it was also shown to produce an unexpected 13% decrease in calcium uptake, an observation that remained unexplained (24). Therefore, initially we determined the effects of jasmonol on calcium uptake in skeletal muscle and cardiac SR membranes. A concentration-dependent increase in calcium uptake, reaching a maximum of about 45% at 1 μM jasmonol, was observed in both types of microsomes when tested under our standard assay conditions (Fig. 1). Jasmonol produced no effect on the ATP-regenerating system used in the assay, and the regenerating system was fully effective in maintaining the specified ATP concentrations.

Effect of Jasmonol on Calcium Uptake by Trypsin-treated, Phosphorylated, and Control Microsomes—As expected from our previous studies (9), trypsin treatment of cardiac microsomes increased calcium uptake over a range of Ca2+ concentrations extending from 0.02 to 11 μM (Fig. 2A and Table I). Jasmonol (1 mM) increased calcium uptake considerably at the highest Ca2+ concentrations tested (>1 μM) with little effect at the lower Ca2+ concentrations. The Vmax(Ca) of calcium uptake was significantly increased by trypsin treatment and to a greater extent by jasmonol treatment. The largest increase in calcium uptake was produced by the combination of trypsin treatment and jasmonol, which appeared to act additively. Trypsin treatment of microsomes reduced the Km(Ca) of the calcium pump by 10%, whereas jasmonol increased it both in control and trypsin-treated microsomes by 71 and 84%, respectively, and, unlike trypsin, slightly reduced the Hill coefficient.

Phosphorylation of microsomes increased the Vmax(Ca) of the calcium pump by 32% and decreased Km(Ca) by 20% (Fig. 2B and Table I). As was observed in trypsin-treated microsomes,
Jasmone increased both $V_{\text{max}}(\text{Ca})$ and $K_m(\text{Ca})$ in phosphorylated microsomes and their controls (Table I). Phosphorylation of our purified microsomes by the isolated catalytic subunit of PKA resulted in an increase in calcium uptake that was virtually identical to the increase obtained with the holoenzyme. At 0.32 mM Ca$^{2+}$, the rates of calcium uptake by microsomes phosphorylated with the PKA catalytic subunit and by control microsomes were 0.23 ± 0.03 and 0.16 ± 0.02 μmol mg$^{-1}$ min$^{-1}$ (mean ± S.E., n = 3), respectively, compared with rates of 0.22 and 0.15 μmol mg$^{-1}$ min$^{-1}$, respectively, in microsomes phosphorylated with the holoenzyme (data taken from Fig. 2B), an approximately 45% increase with phosphorylation. At 11 μM Ca$^{2+}$ when the catalytic subunit was used, the calcium uptake rates were 0.54 ± 0.10 and 0.41 ± 0.07 μmol mg$^{-1}$ min$^{-1}$ in phosphorylated and control microsomes, respectively, compared with rates of 0.47 and 0.36 μmol mg$^{-1}$ min$^{-1}$, respectively, when the holoenzyme was used (data taken from Fig. 2B), both displaying an increase in calcium uptake of approximately 30% with phosphorylation. The differences between the microsomes phosphorylated with the PKA catalytic subunit and their controls were statistically significant at $p < 0.05$ when tested by Student’s $t$ test for paired variates.

The effects of phosphorylation (or trypsin treatment) on our purified microsome preparations stand in sharp contrast to the results obtained by most laboratories that have utilized crude cardiac microsomes (e.g. Ref. 6), cardiac tissue homogenates (4), reconstituted vesicles containing purified Ca$^{2+}$-ATPase and PLN (16), or crude microsomes isolated from noncardiac cells with expressed proteins (11). In these preparations, phosphorylation affects exclusively or predominantly $K_m(\text{Ca})$, while changes in $V_{\text{max}}(\text{Ca})$ are generally of small magnitude (6), highly variable among different preparations (cf. Refs. 6 and 9), or absent altogether (e.g. Refs. 4, 11, and 16). Moreover, $V_{\text{max}}(\text{Ca})$ can be increased in purified cardiac microsomes by means other than phosphorylation, in this case by jasmone.

Calcium Uptake Assayed at 0–10 μM Mg-ATP—In contrast to its effects on calcium uptake measured at 1 mM Mg-ATP, 1 mM jasmone markedly decreased calcium uptake at 0–10 μM Mg-ATP in both control and trypsin-treated microsomes (Fig. 3). In the presence of jasmone, the apparent $V_{\text{max}}(\text{Mg-ATP})$ derived from data in the 0–2 μM Mg-ATP concentration range, which reflects nucleotide binding to the catalytic site, decreased considerably in the control microsomes and to an even greater extent in the trypsin-treated microsomes (Table II). On the other hand, the apparent $K_m(\text{Mg-ATP})$ was increased by jasmone in the control but not in the trypsin-treated microsomes. Between 2 and 10 μM Mg-ATP, the degree of inhibition by jasmone...
Jasmone and Phospholamban Action on the Cardiac SR Calcium Pump

Kinetic parameters for calcium uptake by trypsin-treated, phosphorylated, and control microsomes measured at 25 °C in the presence and absence of 1 mM jasmone

| Microsomes      | Jasmone | $V_{max(Ca)}$ (μmol mg⁻¹ min⁻¹) | $K_{m(Ca)}$ (μM) | Hill coefficient |
|-----------------|---------|---------------------------------|------------------|-----------------|
| Group 1         |         |                                 |                  |                 |
| Control         | −       | 0.43 ± 0.06a,b                 | 100              | 1.71 ± 0.08b    | 100             |
| Control         | +       | 0.62 ± 0.09a                 | 144              | 1.44 ± 0.06b    | 84              |
| Trypsin-treated | −       | 0.58 ± 0.09c,d              | 135 (100)        | 1.67 ± 0.14c    | 98 (100)        |
| Trypsin-treated | +       | 0.82 ± 0.11c,d              | 191 (141)        | 1.46 ± 0.12c    | 85 (87)         |
| Group 2         |         |                                 |                  |                 |
| Control         | −       | 0.53                           | 100              | 1.62            | 100             |
| Control         | +       | 0.77                           | 145              | 1.66            | 102             |
| Phosphorylated  | −       | 0.70                           | 132 (100)        | 1.70            | 105 (100)       |
| Phosphorylated  | +       | 0.87                           | 164 (124)        | 1.44            | 89 (85)         |

a,b,c,d Differences between the values identified by the same superscript in each column are significant at $p < 0.05$ when tested by Student's $t$ test for paired variates.

Ca²⁺-ATPase Activity and Calcium Uptake: Temperature Effects—We carried out experiments at 37 °C in addition to those at 25 °C in order to determine the temperature dependence of some of the calcium pump properties. No correction for the change in temperature is necessary in deriving Ca²⁺ concentrations, since temperature has been shown not to have a significant effect on the Ca²⁺ concentration established by a CaCl₂-EGTA buffer system (30). Therefore, the same CaCl₂-EGTA buffer systems were used except that the pH was adjusted for the higher temperature. However, the solubility product of calcium oxalate increases with temperature, resulting in higher intraluminal Ca²⁺ at constant oxalate concentration. Because of uncertainty about the value of the solubility product appropriate for the intraluminal environment, an initial set of experiments was carried out at 2.5 mM oxalate, as before, followed by experiments at 5.0 mM oxalate. In the measurements of ATPase activity below, this problem is avoided altogether by the use of a Ca²⁺ ionophore.

At 37 °C in the presence of 2.5 mM oxalate and 11 μM Ca²⁺, calcium uptake rates by control microsomes (treated with trypsin inhibitor-inactivated trypsin) were 1.37 ± 0.06 and 1.38 ± 0.07 μmol mg⁻¹ min⁻¹ in the absence and presence, respectively, of 1 mM jasmone. In trypsin-treated microsomes, the rates were 1.73 ± 0.16 and 1.47 ± 0.13 μmol mg⁻¹ min⁻¹, respectively, in the absence and presence of jasmone. Thus, unlike our findings at 25 °C, at 37 °C jasmone not only failed to stimulate calcium uptake in both control and trypsin-treated microsomes but, in fact, significantly decreased it in the trypsin-treated microsomes ($p < 0.05$) by 15%. Trypsin treatment of microsomes, nevertheless, stimulated calcium uptake by 26% in this series of experiments ($p < 0.05$).

In order to establish whether protein kinase A-catalyzed phosphorylation of microsomes or jasmone modified calcium uptake via an effect on the calcium pump protein rather than by a general perturbation of membrane permeability, we assayed the Ca²⁺-ATPase activity in our purified microsomes under various conditions (Table III). No ATPase activity attributable to the microsomes was detectable in 10 mM EGTA either at 25 or 37 °C irrespective of whether the microsomes had been phosphorylated by PKA or incubated under control conditions (see “Experimental Procedures”). This finding as well as a lack of ouabain-sensitive Na⁺,K⁺-ATPase activity suggests an absence of significant contamination of our purified cardiac microsomes by plasma membranes.

At 25 °C, phosphorylation increased Ca²⁺-ATPase activity...
assayed at 11 μM (saturating) Ca\(^{2+}\) by 38% and by 25% at 0.32 μM Ca\(^{2+}\) (Table III), which is close to the \(K_m\) (Ca) of calcium uptake (see Table I). These increases in ATPase activity are similar to the phosphorylation- or trypsin-induced increases in calcium uptake seen at 25°C in Fig. 2 and Table I. At 37°C, phosphorylation increased calcium uptake and Ca\(^{2+}\)-ATPase activity assayed at 0.32 μM Ca\(^{2+}\) by 91 and 100%, respectively, and at 11 μM Ca\(^{2+}\) by 21 and 18%, respectively. Comparable increases in calcium uptake upon phosphorylation were observed when the assays were carried out at 5.0 mM instead of 2.5 mM oxalate. Therefore, both trypsin treatment and phosphorylation of purified cardiac microsomes enhance calcium pump activity also at 37°C and 11 μM Ca\(^{2+}\) under the conditions employed. As expected on the basis of our measurements of calcium uptake, jasmone increased Ca\(^{2+}\)-ATPase activity measured at 25°C and 11 μM Ca\(^{2+}\) beyond the increase produced by phosphorylation, whereas at 37°C, jasmone produced a slight decrease in control Ca\(^{2+}\)-ATPase activity (Table III), which, although only 3%, was statistically significant at the 0.05 level when tested by Student’s t test for paired variates.

### Table II

**Apparent kinetic parameters for calcium uptake measured at 0–2 μM Mg-ATP and 25°C in the presence and absence of 1 mM jasmone**

| Microsomes          | Jasmone | \(V_{\text{max}}\) (Mg-ATP) | \(K_m\) (Mg-ATP) |
|---------------------|---------|-----------------------------|------------------|
| Control             | –       | 0.12                        | 100              |
| Control             | +       | 0.05                        | 42               |
| Trypsin-treated     | –       | 0.15                        | 125 (100)        |
| Trypsin-treated     | +       | 0.03                        | 25 (20)          |

When assayed at 23°C under our standard assay conditions (Table IV), the addition of jasmone to trypsin-treated microsomes produced a further 30% reduction, suggesting that at 23°C the effects of trypsin treatment and jasmone act additively. However, when the microsomes were assayed at 37°C, no significant decreases in \(E_P\) levels were detected as a result of either trypsin treatment or jasmone, whether alone or in combination.

We therefore predicted that the effects of jasmone and trypsin on steady-state \(E_P\) levels would be even more pronounced at 15°C than at 23°C, since \(E_P\) decomposition in untreated (control) microsomes becomes slower at lower temperatures and hence would be more susceptible to acceleration. As seen in Table IV, jasmone and trypsin treatment of microsomes each decreased steady-state \(E_P\) levels to about 55% of the control value, and jasmone added to trypsin-treated microsomes reduced them still further to 42%. Steady-state \(E_P\) formation from P in control microsomes remained essentially unchanged in the temperature range tested.

**DISCUSSION**

In this report evidence is presented for a role of PLN in the regulation of cardial SR Ca\(^{2+}\)-ATPase that may contribute to or account for an observed increase in \(V_{\text{max}}\) (Ca) and decrease in \(K_m\) (Ca) of microsomal calcium uptake upon PKA-catalyzed PLN phosphorylation (Fig. 2 and Table I). The first step implicated in this regulation is step 2 or 2a in Scheme 1 or step 1c in Scheme 2, which explicitly lists additional putative substeps in the formation of \(E_1\)-ATP-2Ca from \(E_0\) (19, 34, 35). The second is step 7 or \(E_P\) decomposition. Step 1c in Scheme 2, which is shown in the presence of (Mg-)ATP as in Ref. 19, represents a conformational change in the Ca\(^{2+}\)-ATPase protein upon binding of the first of two Ca\(^{2+}\). Direct evidence for PLN’s inhibition of the rate of this conformational change was provided by Cantilina et al. (12), who associated this kinetic effect with a shift in the \(K_m\) (Ca) of calcium uptake; such a shift was not seen in measurements of equilibrium binding of Ca\(^{2+}\) in the absence of ATP.

Measurement of the rate of \(E_P\) decomposition in the present study (Fig. 4) indicates that also this step in the cycle can be accelerated by jasmone or removal of PLN’s inhibitory influence by trypsin treatment of the microsomes. This observation is consistent with the decrease in steady-state \(E_P\) levels when the microsomes are incubated in the presence of P, at 15 and 25°C (Table III). This result represents an advance over an earlier study with crude microsomes (36), which showed that Ca\(^{2+}\)-ATPase turnover is accelerated upon microsomal phosphorylation as a result of an increase in “EP” breakdown but did not distinguish between \(E_P\) and \(E_P\). On the other hand, Cantilina et al. (12) failed to observe any increase in the rate of decay of phosphoenzyme formed from ATP as a result of incubation of microsomes in the presence of monoclonal antibody against PLN. The reason for this discrepancy is not readily apparent. Our demonstration of effects of PLN on the rate of \(E_P\) decomposition and steady-state \(E_P\) formation from P,
TABLE III
Calcium uptake in phosphorylated and control microsomes

| Temperature | PKA | Jasmone | Ca\(^{2+}\)-ATPase activity (0.32 \(\mu\)mol Ca\(^{2+}\)) | Ca\(^{2+}\)-ATPase activity (11 \(\mu\)mol Ca\(^{2+}\)) |
|-------------|-----|---------|----------------------------------|----------------------------------|
| 25 °C       | –   | –       | 0.08 ± 0.00                      | 0.29 ± 0.01                       |
|             | +   | –       | 0.10 ± 0.00                      | 0.54 ± 0.01                       |
|             | +   | +       | 0.91 ± 0.09                      | 133                              |
| 37 °C       | –   | –       | 0.16 ± 0.01                      | 1.20 ± 0.13                      |
|             | +   | –       | 0.32 ± 0.02                      | 1.42 ± 0.12                      |
|             | +   | +       | 1.38 ± 0.06                      | 15                               |

Purified cardiac microsomes were incubated at the indicated temperature in the presence (+) and absence (–) of 0.11 mg/ml protein kinase A (PKA) and 2 \(\mu\)M cyclic AMP under conditions favorable for phosphorylation followed by the addition of either 1 mm jasmone or an equivalent volume of distilled water as indicated. Ca\(^{2+}\)-ATPase and calcium uptake reactions were carried out in the presence of 5.0 mM oxalate-Tris and 0.3 mg/ml PKA, as indicated. The values are the means ± S.E. of three independent experiments with different microsome preparations except as indicated. Differences between the control and each other value under a particular set of conditions are significant at the \(p < 0.05\) level when tested by Student’s t test for paired variates.

Ca\(^{2+}\)-ATPase activity and calcium uptake in phosphorylated and control microsomes

| Temperature | PKA | Jasmone | Calcium uptake (0.32 \(\mu\)mol Ca\(^{2+}\)) | Calcium uptake (11 \(\mu\)mol Ca\(^{2+}\)) |
|-------------|-----|---------|----------------------------------|----------------------------------|
| 37 °C       | –   | –       | 0.47 ± 0.04                      | 1.91 ± 0.18                      |
|             | +   | –       | 0.90 ± 0.05                      | 2.32 ± 0.22                      |

Mean of four experiments.

A second major factor contributing to the contradictory results among laboratories is the use of different preparations of calcium pump protein and incubation conditions. One of the recent reports that concluded that PLN has no effect on \(V_{\text{max}}\) (Ca\(^{2+}\)) of calcium uptake was based on experiments utilizing purified Ca\(^{2+}\)-ATPase and PLN reconstituted into phospholipid-containing vesicles (16). Reconstitution of vesicles represents a modification of the native SR membrane that may eliminate factors necessary for normal protein interaction or function. A case in point is the discovery that reconstitution of purified skeletal muscle Ca\(^{2+}\)-ATPase into vesicles containing phosphatidylinositol 4-phosphate leads to a doubling of the rate of \(E_{2}P\) decomposition with a corresponding decrease in steady-state \(E_{2}P\) formation from \(P_{2}\), whereas no changes are observed in the phosphorylation of the enzyme by ATP or in the transport step (37). Furthermore, the purification and reconstitution procedures utilized by Reddy et al. (16) rely on the use of nonionic detergents, which have been shown to interfere specifically with the demonstration of changes in the \(V_{\text{max}}\) (Ca\(^{2+}\)) of the cardiac calcium pump following trypsin treatment when present at micromolar concentrations (9). The use of Bio-Beads by Reddy et al. to remove detergents from the reconstituted vesicles is based on a competition between the Bio-Beads and protein for the detergent and may not be fully effective with cardiac Ca\(^{2+}\)-ATPase when utilizing the procedures that have...
Effect of temperature on steady-state \( E_P \) formation from \(^{32}P \) in control and trypsin-treated microsomes

| Microsomes          | 15 \(^{\circ}\)C | \( \frac{\text{nmmol} E_P \text{P/mg}}{\text{mg}} \) | %        | 23 \(^{\circ}\)C | \( \frac{\text{nmmol} E_P \text{P/mg}}{\text{mg}} \) | %        | 37 \(^{\circ}\)C | \( \frac{\text{nmmol} E_P \text{P/mg}}{\text{mg}} \) | %        |
|---------------------|-----------------|------------------|---------|-----------------|------------------|---------|-----------------|------------------|---------|
| Control             | 1.27 ± 0.04**   | 100              | 1.34 ± 0.07*** | 100              | 1.28 ± 0.10      | 100     |
| Trypsin-treated     | 0.70 ± 0.06***  | 55               | 0.96 ± 0.07**  | 72               | 1.29 ± 0.11      | 101     |
| Trypsin-treated     | 0.73 ± 0.03***  | 57 (100)         | 0.98 ± 0.02**  | 73 (100)         | 1.23 ± 0.09      | 96 (100) |

Note: The data shown in Fig. 9 of Starling et al. (24), indicating that jasmonate does not perturb the pH dependence of the fluorescence of 4-nitrobenzo-2-oxa-1,3-diazole-labeled Ca\(^{2+}\)-ATPase, which is believed to monitor the \( E_1 \leftrightarrow E_2 \) equilibrium in the absence of Ca\(^{2+}\). Since \( \mu M \) Mg-ATP is known to accelerate the \( E_2 \rightarrow E_1 \) and \( E_1 \rightarrow 1Ca \rightarrow E_1 \rightarrow 1Ca \) transitions (19, 32), the proposed inhibition of the latter step by jasmonate may be much reduced but not eliminated at higher Mg-ATP concentrations (see below).

At 37 \(^{\circ}\)C and millimolar ATP on the other hand, both \( V_{\text{max}}(\text{Ca}) \) of calcium uptake (estimated at 11 \( \mu M \) Ca\(^{2+}\); see "Results") and the steady-state level of \( E_P \) formation (Table IV) are unaffected by jasmonate. Although the rate of \( E_P \) decomposition was too rapid to measure with the QFM-5 rapid mixing system at 37 \(^{\circ}\)C, the steady-state \( E_P \) formation from \( P_i \) which reflects changes in \( E_P \) decomposition relative to its formation (24, 32), was unaffected by jasmonate, trypsin, or both (Table IV). Trypsin treatment or phosphorylation, however, produced a significant increase in calcium uptake (see "Results") or Ca\(^{2+}\)-ATPase activity (Table III), respectively, when measured at 37 \(^{\circ}\)C. Thus, increasing the temperature from 25 to 37 \(^{\circ}\)C may shift the putative rate-limiting step from strongly temperature-dependent \( E_P \) decomposition (33) to the next slowest step in the cycle, which has a weaker temperature dependence and is blocked by PLN. In this case, candidates for the next slowest step in the cycle are the \( E_2 \rightarrow 1Ca \rightarrow E_1 \) transition (step 1c or 1e in Scheme 2) or the \( E_2 \rightarrow 2Ca \rightarrow E_3 \) transitions (steps 5 and 6 in Scheme 1). The latter step was suggested by Hughes et al. (39) to be regulated by PLN on the...
basis of a study with a synthetic peptide analog of PLN. The proposed inhibition by jasmone of the \( E_2 \rightarrow E_1 \cdot P \cdot 2 \text{Ca} \) transition or, specifically, the \( E_1 \cdot \text{Ca}_1 \rightarrow E_1' \cdot \text{Ca}_1 \) transition (12) could explain the 15% inhibition of calcium uptake that was produced by jasmone in trypsin-treated microsomes (see “Results”) and the smaller yet statistically significant decrease in \( \text{Ca}^{2+} \) produced by jasmone in trypsin-treated microsomes (see “Results”) and the smaller yet statistically significant decrease in \( \text{Ca}^{2+} \)-ATPase activity (Table IV), although other interpretations are possible. Thus, at 37 °C, when whole cycle turnover is accelerated by temperature, trypsin treatment, or phosphorylation (Table III) and the stimulatory actions of jasmone on \( E_2 \cdot P \) decomposition (Fig. 4 and Table IV) and on the \( E_1' \cdot P \rightarrow E_2 \cdot P \) transition (24), the proposed inhibition by jasmone of the \( E_1' \cdot \text{Ca}_1 \rightarrow E_1' \cdot \text{Ca}_1 \) transition, as discussed further below, may again become apparent.

Multiple additional factors other than the ones explored in the present study, such as pH and the intraluminal environment, are likely to impinge upon the different steps in the catalytic cycle of the \( \text{Ca}^{2+} \)-ATPase and affect their contribution to rate limitation under physiological conditions. However, the potential for regulation of both \( V_{\text{max}} (\text{Ca}) \) and \( K_m (\text{Ca}) \), of the SR calcium pump by PLN that we have demonstrated is likely to be of physiological significance in the event of attainment of saturating intracellular \( \text{Ca}^{2+} \) concentrations at peak systole as a result of strong \( \beta \)-adrenergic stimulation of the heart or under pathologic conditions involving calcium overload. Under such conditions, an effect of \( \beta \)-adrenergic agonist-induced PLN phosphorylation on both kinetic parameters will ensure abbreviation of systole at high heart rates, promote greater filling of SR \( \text{Ca}^{2+} \) stores (40), and increase the rate of myocardial relaxation to promote diastolic filling.

Acknowledgment—We thank Dr. Massimo Sassaroli for helpful discussions and critical review of the manuscript.

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