# Concerted Phosphorylation of the 26-Kilodalton Phospholamban Oligomer and of the Low Molecular Weight Phospholamban Subunits†

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ABSTRACT: Phospholamban (PLB) from cardiac sarcoplasmic reticulum (SR) was phosphorylated under various conditions by the adenosine cyclic 3′,5′-phosphate (cAMP)-dependent and/or the calmodulin-dependent protein kinase. The small shifts in apparent molecular weight resulting from the incorporation of $P_i$ groups in the PLB complexes were analyzed by high-resolution sodium dodecyl sulfate–polyacrylamide gel electrophoresis. In parallel experiments, PLB was dissociated into its subunits and analyzed by using a newly developed isoelectric focusing system. The p*I* values of the PLB subunits phosphorylated by the cAMP- or calmodulin-dependent kinase were 6.2 and 6.4, respectively. Double phosphorylation of the same subunit resulted in an acidic shift of the p*I* to 5.2. The combined analysis of the behavior of the PLB complex and of its subunits has greatly simplified the interpretation of the complex phosphorylation pattern and has led to the following conclusions: (i) The PLB complex is composed of five probably identical subunits, each of them containing a distinct phosphorylation site for the calmodulin- and the cAMP-dependent kinase. (ii) The population of PLB interacting with the endogenous calmodulin-dependent kinase cannot be phosphorylated by the cAMP-dependent kinase unless previously phosphorylated in the presence of calmodulin. It was also observed that after maximal phosphorylation of PLB in the presence of very large amounts of the cAMP-dependent protein kinase, the $Ca^{2+}$ pumping rate of the cardiac SR ATPase is stimulated up to 5-fold, i.e., a level of a stimulation which exceeds considerably the values so far reported in the literature.

The ($Ca^{2+}$, $Mg^{2+}$)-ATPase of skeletal and heart sarcoplasmic reticulum (SR)[1] reduces the free $Ca^{2+}$ concentration in the sarcoplasm to the submicromolar range in the resting muscle by pumping the cytosolic calcium into the cavities of the SR. The regulation of the ATPase has been the subject of intensive studies. Phospholamban (PLB), a protein component of the SR membrane of heart muscle, was found to play a key role in the regulation of the ATPase (Wray et al., 1973; Katz & Remtulla, 1978). Several reports have demonstrated that phosphorylated PLB markedly stimulates the $Ca^{2+}$ pump. Phosphorylation may be brought about by a cAMP-dependent protein kinase (cAMP-PK) (Kirchberger et al., 1974; Schwartz et al., 1976; Will et al., 1978; Tada et al., 1978) and/or by a $Ca^{2+}$–calmodulin-dependent protein kinase (Calm-PK) (Le Peuch et al., 1979; Kirchberger & Antonetz, 1982). A third kinase, identified as protein kinase C, has recently also been shown to phosphorylate PLB (Movsesian et al., 1984). An endogenous phosphatase has been claimed to dephosphorylate phosphorylated PLB (Tada et al., 1975; Le Peuch et al., 1979) and to decrease the initial rate of $Ca^{2+}$ transport by the ATPase (Kranias, 1985a). Phosphorylation–dephosphorylation is therefore a plausible regulation mechanism for the ATPase.

Little is known on the molecular structure of PLB. Attempts to characterize the molecular weight, the subunit composition, and the physicochemical properties of PLB have led to controversial results. The protein was originally described as a species having a molecular weight in the range 23K–28K, but fully dissociated purified PLB was described as a single SDS–PAGE band of apparent $M_r$ 11K (Inui et al., 1985) or as a lower band of $M_r$ 5.5K (Kirchberger & Antonetz, 1982). p*I* determinations have varied from 3.7 (Le Peuch et al., 1980; Capony et al., 1983) to 10 (Jones et al., 1985). Marked differences in the amino acid composition have also been reported (Le Peuch et al., 1980; Bidlack et al., 1982; Capony et al., 1983).

Phosphorylation by either the cAMP- or the calmodulin–$Ca^{2+}$-dependent kinase results in a shift of the PLB complex to higher apparent molecular weight on SDS gels (Wegener & Jones, 1984; Imagawa et al., 1986). A detailed analysis of the shift has led to the suggestion of multiple phosphorylation sites in the PLB complex (Jones et al., 1985): Very recently, five distinct phosphorylation products were detected after phosphorylation of PLB cy the cAMP-PK (Imagawa et al., 1986), supporting the suggestion of PLB as a pentamer of five subunits having an apparent molecular weight of about 5K (Wegener & Jones, 1984; Imagawa et al., 1986). Previous work from this laboratory has suggested two functionally distinct PLB complexes, selectively phosphorylated by the Calm-PK or the cAMP-PK (Chiesi et al., 1983). In the present work, an analytical isoelectric focusing system (IEF) has been used which has made possible the study of the PLB subunits after phosphorylation under different conditions. The results have provided support for the above-mentioned suggestion of a possible functional difference between the PLB

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[1] Abbreviations: SR, sarcoplasmic reticulum; PLB, phospholamban; cAMP, adenosine cyclic 3′,5′-phosphate; SDS, sodium dodecyl sulfate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; Chaps, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; $Me_2SO$, dimethyl sulfoxide; kDa, kilodalton(s); cAMP-PK, cAMP-dependent protein kinase; Calm-PK, $Ca^{2+}$–calmodulin-dependent protein kinase; IEF, isoelectric focusing; Tris, tris(hydroxymethyl)aminomethane; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

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phosphorylated by the Calm-PK and that phosphorylated by the cAMP-PK.

## Materials and Methods

Dog hearts were obtained from pentothal-anesthetized dogs from the University Hospital, Zurich. [$\gamma$-$^{32}$P]ATP was purchased from Amersham International, U.K.; the catalytic subunit of bovine heart cAMP-dependent protein kinase and Chaps were from Sigma Chemical Co., St. Louis, MO; carrier ampholines and agarose Z (low endoelectroosmotic) were from LKB-Produkter AB, Bromma, Sweden; low molecular weight marker proteins were from Bio-Rad Chemical Division, Richmond, CA; Millipore 22-$\mu$m filters (type HA) were from Millipore Corp., Bedford, MA; calmodulin was the generous gift of Dr. D. Guerini, Zurich. All other chemicals were of analytical grade.

*Preparation of Cardiac SR Vesicles.* Cardiac SR was prepared essentially as described by Chamberlain et al. (1983), but some modifications were introduced: dog heart ventricles were cut into small pieces and rinsed in ice-cold 0.154 M NACl. To about 100 g of trimmed tissue, 350 mL of 0.29 M sucrose, 10 mM imidazole, pH 6.9, 0.5 mM DTT, and 0.1 mM PMSF in $Me_2SO$ (=medium I) were added. A first homogenization was performed in a Waring Blendor (10 times for 2 s). A second homogenization was performed in a Buehler-type UMS homogenizer for 10 and 25 s at full speed with a 5-s break in between. The second homogenization was performed in four portions. The homogenate was centrifuged for 5 min at $500g_{max}$ in a Sorvall GSA rotor to obtain the postnuclear supernatant. The latter was filtered through hydrophilic gauze, and fresh medium I was added to adjust the total volume to 350 mL. A second centrifugation was performed for 15 min at $38000g_{max}$. After filtration of the resulting supernatant, a third centrifugation (10 min at $8000g_{max}$) was performed. The resulting pellets were resuspended in 5 mL of medium I and kept as the mitochondrial fraction. The supernatant was filtered again through gauze and centrifuged for 15 min at $23000g_{max}$. The pellet from this centrifugation was resuspended in 5 mL of medium I and kept as the heavy crude SR fraction. The supernatant was then centrifuged for 60 min at $120000g_{max}$ in a Beckman TI 35 rotor. The resulting pellets were resuspended in 50 mL of medium I containing in addition 0.65 M KCl (=medium II) and incubated for 30 min on ice with slow stirring. Centrifugation for 10 min at $4400g_{max}$ in a Sorvall SS 34 rotor removed aggregated material. The final SR fraction was obtained by a second high-$g$ centrifugation (60 min at $250000g_{max}$) in a Beckman TI 60 rotor. The resulting pellet was resuspended in 10 mL of medium I and quickly frozen in liquid nitrogen before storage at −80 °C.

*Miscellaneous Methods.* The SR membranes were characterized as follows: mitochondrial contamination was determined by measuring the cytochrome $c$ oxidase activity polarographically with a Clark-type oxygen electrode (Warton & Griffiths, 1962); contamination with sarcolemmal membranes was assayed by determination of [$^3$H]ouabain binding (Lau et al., 1979); maximal $Ca^{2+}$-dependent ATPase activity was measured in the presence of 1 $\mu$g/mL ionophore A 23187 at 37 °C by a coupled enzyme assay (Niggli et al., 1979). The ATPase content of the SR membranes was estimated by denstiometric scanning of Coomassie brilliant blue stained SDS–PAGE in a Shimadzu dual-wavelength scanner. The protein was measured according to the method of Lowry et al. (1951) with bovine serum albumin as a standard.

*$Ca^{2+}$ Uptake.* $Ca^{2+}$ uptake was measured with the Millipore filtration technique, essentially as described by Chamberlain et al. (1983); 22-$\mu$m Millipore filters, type HA, were used. All experiments were carried out at 37 °C. The incubation medium contained 100 mM KCl, 100 mM sucrose, 20 mM imidazole, pH 7.0, 5 mM $MgCl_2$, 5 mM $NaN_3$, 500 $\mu$M EGTA, and the amount of $CaCl_2$ required to produce the desired free concentration of 0.5 $\mu$M. The latter was calculated with the program of Fabiato and Fabiato (1979), adapted to an Apple IIe computer. Standard uptake experiments were performed as follows: 10 $\mu$g/mL SR vesicles were incubated for 4 min at 37 °C in the incubation medium containing 100 mM sucrose, 100 mM KCl, 20 mM imidazole, pH 7.0, 5 mM $MgCl_2$, 5 mM $NaN_3$, 500 $\mu$M EGTA, 272 $\mu$M $CaCl_2$, and 0.5 mM $Na_2ATP$, with or without 6 $\mu$M calmoudlin and different amounts of the catalytic subunit of the cAMP-dependent protein kinase (cAMP-PK). $Ca^{2+}$ upake was started by adding $Na_2ATP$ and potassium oxalate to produce final concentrations of 5 and 3 mM, respectively. The uptake reaction was quenched by filtering the aliquots withdrawn and by four washes of the filters with 0.5 mL of ice-cold 100 mM KCl, 100 mM sucrose, 20 mM imidazole, pH 7.0, 5 mM $MgCl_2$, 5 mM $NaN_3$, and 3 mM $LaCl_3$. The filters were counted in 3 mL of Packard 299 scintillation cocktail in a Beckman liquid scintillation counter.

*SDS–PAGE.* Routinely, 15% T/0.4% C Laemmli gels were used. To obtain higher resolution, the gels were allowed to stand at room temperature for at least 24 h to obtain complete polymerization. Such gels were run at lower currents (constant current of 20 mA). Standard gels were polymerized for only 1 h at room temperature and run routinely at 35-mA constant current; 5–30 $\mu$g of SR protein was loaded. Gels were fixed in 50% methanol and 10% TCA for 30 min and stained in 20% methanol, 7.5% acetic acid, and 0.25% Serva Blau R 250. When required, the gels were dried and autoradiographed with Kodak X-Omat AR films for 24 h without an amplifying screen. Molecular weight determination was achieved by calibrating the gels with various mixtures of marker proteins. The following proteins were used: (2) low molecular weight standards from Bio-Rad Chemical Division; (ii) a homemade mixture containing bovine serum albumin, aldolase, chymotrypsinogen, and cytochrome $c$, and (iii) aprotinin, glucagon, and insulin for the range below 10 000 Da.

*Phosphorylation of SR Vesicles.* Standard phosphorylations were carried out in a phosphorylation medium containing 100 mM NaCl, 5 mM $MgCl_2$, 20 mM Hepes, pH 7.4, 1000 $\mu$M EGTA, and 900 $\mu$M $CaCl_2$; 0.3–1.0 mg/mL SR protein was preincubated for 5 min at room temperature in the phosphorylation medium in the presence, when required, of 6 $\mu$M calmodulin and 60–600 units/mL cAMP-PK. The phosphorylation reaction was started by adding [$\gamma$-$^{32}$P]ATP to a final concentration of 300 $\mu$M at a specific activity of 0.05–0.5 $\mu$Ci/nmol. The phosphorylation was stopped at the times indicated with SDS sample buffer containing 2.5% SDS (final concentration).

*Pulse–Chase Phosphorylation.* In some experiments, the phosphorylation was started by adding labeled ATP to 100 nM final concentration, followed by the addition, within 2 s, of cold ATP to 300 $\mu$M final concentration.

*Prephosphorylation.* When required, SR vesicles were prephosphorylated under the conditions described above. At the desired reaction times, the phosphorylation reaction was stopped by adding NaF, $NaP_i$, and Na-EDTA to final concentrations of 25 mM, 10 mM, and 10 mM, respectively. The samples were immediately centrifuged at 4 °C in a Beckman airfuge for 10 min at $150000g_{max}$. The resulting pellets were resuspended with a homemade micro Teflon pestle directly

in the airfuge tubes. Subsequent phosphorylations under various conditions were performed on this material.

The sample preparation for isoelectric focusing experiments differed slightly: The phosphorylation reactions were performed under the conditions described above. After the desired phosphorylation times, the reactions were stopped by adding 1 volume of IEF sample buffer, containing 4% w/v Triton X-100, 2% w/v Chaps, 2% w/v carrier ampholine, 2% v/v 2-mercaptoethanol, and a trace of bromophenol blue. The samples were immediately boiled for 5 min in a water bath, transferred into airfuge tubes, and centrifuged for 10 min at $150000g_{max}$ in the airfuge. The resulting supernatants were used for the isoelectric focusing gels.

*Isoelectric Focusing on Agarose Gels.* One percent w/v agarose Z IEF gels (12.5 × 11 cm) were cast in an LKB ultramold on plastic film (LKB). Agarose was found to be much more suitable than polyacrylamide, because it allows the use of different detergents and its bed has a fairly large pore size, minimizing artifacts induced by the migration of protein–detergent complexes. The gel solution contained 1% w/v agarose Z, 2% w/v Triton X-100, 1% w/v Chaps, 2% carrier ampholine of the desired pH range, 10% sorbitol, and 20% glycerol. The gels were prefocused at 10 °C for 1 h at 2.5-W constant power with an LKB microdrive power supply. The anode strip contained 50 mM $H_2SO_4$, the cathode strip 1 M NaOH. After prefocusing was completed, excess water was removed from the gel surface with Whatman filter paper, and then the samples were loaded. They were applied with a Hamilton Syringe in an LKB Mylar application foil. The gels were routinely focused for 2.5 h at 2.5-W constant power. Hemoglobin lysate was used as a colored marker to follow the focusing process. pH calibration was performed by two different methods: p*I* marker proteins from Pharmacia were run on each gel. Manual calibration was done by cutting 0.5 × 1 cm pieces immediately after the end of the run. These pieces were incubated for 30 min in 1 mL of triple-distilled water, and the pH was measured with a glass electrode. The gradient stability was controlled with the same method. After being completely focused, the gels were routinely transferred to 100 mL of 30% v/v ethanol and 10% w/v TCA for 30 min. The resulting white precipitate was washed out from the gels by incubating them overnight in 30% ethanol and 5% TCA. Before being dried, the gels were briefly rinsed in 20% methanol and 7.5% acetic acid. Drying and staining of the gels were routinely performed as described by Pharmacia Fine Chemicals (1982). Autoradiography was performed as described above for the Laemmli gels.

When required, semiquantitative analysis of the phosphoproteins was performed by cutting the interesting bands from the dry gel and by counting them in 3 mL of Packard 299 scintillation cocktail in a Beckman liquid scintillation counter.

For two-dimensional electrophoresis, the strips were cut out together with the gel bond film from the IEF gel immediately after completion of focusing. Two different treatments were used prior to performing the second dimension: (1) the strips were treated as described above (fixing and washing overnight); (2) the strips were incubated for 20 min in 10 mL of equilibration buffer containing 60 mM Tris, pH 6.8, 5% v/v 2-mercaptoethanol, 3.3% w/v SDS, and 0.01% bromophenol blue. After such treatment, the strips were layered on top of a 1.5-mm-thick % stacking gel and embedded with 1% melted agarose in 4 times diluted stacking gel buffer, pH 6.8. SDS–PAGE was run as described above.

## Results

*Preparation of SR Membranes.* Although the preparation

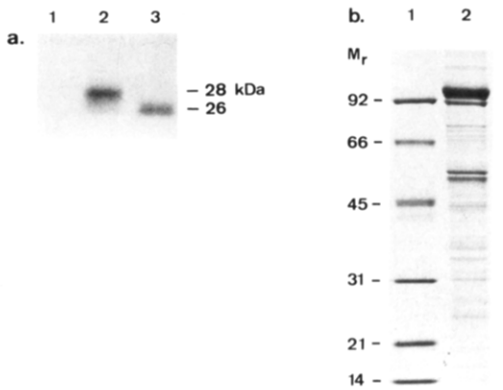


FIGURE 1: (a) Phosphorylation of cardiac SR vesicles under various conditions. Thirty micrograms SR was phosphorylated at a protein concentration of 1 mg/mL under standard conditions (see Materials and Methods) in the presence of no addition (lane 1), 6 $\mu$M calmodulin (lane 2), 60 units/mL cAMP-PK (lane 3), and 300 $\mu$M [$\gamma$-$^{32}$P]ATP for 4 min. The reaction was stopped by adding 0.5 volume of SDS sample buffer to yield a final SDS concentration of 2.5%. After 10-min incubation at room temperature, the samples were separated on a 15% T/0.4% C SDS gel. The front was run out for 2 h at 30-mA constant current. The conditions for autoradiography was described under Materials and Methods. (b) Linear gradient (7–12%) SDS gel stained with Coomassie brilliant blue. Thirty micrograms of a typical SR preparation was applied to lane 2. Lane 1 contained 1 $\mu$L of molecular weight marker proteins.

procedure described by Chamberlain et al. (1983) yielded stable and highly active SR vesicles, several additional low-*g* centrifugation steps were included in this study to improve the $Ca^{2+}$-ATPase content to 42–50% of the total protein in the final SR fraction (Figure 1b) as determined by densitometric scannings of Coomassie blue stained SDS gels. A typical SR preparation was contaminated less than 6% with mitochondria (cytochrome *c* oxidase assay; see Materials and Methods) and less than 5% with sarcolemma ([$^3$H]ouabain binding). Maximal $Ca^{2+}$-dependent ATPase activity was 1.57 ± 0.19 $\mu$mol mg$^{-1}$ min$^{-1}$ when measured in the presence of ionophore A 23187 at 37 °C. $Ca^{2+}$ translocation was measured at 0.5 $\mu$M free $Ca^{2+}$ as described under Materials and Methods and was 0.11 ± 0.02 $\mu$mol mg$^{-1}$ min$^{-1}$. At optimal free $Ca^{2+}$ concentration (5.0 $\mu$M free $Ca^{2+}$), the maximal uptake rates were 0.51 ± 0.05 $\mu$mol mg$^{-1}$ min$^{-1}$. The characteristics above represent mean values for three different SR preparations.

*Phosphorylation of Phospholamban by either the cAMP-Dependent or the $Ca^{2+}$–Calmodulin-Dependent Protein Kinase.* Phosphorylation in the presence of 60 units/mL catalytic subunit of cAMP-dependent protein kinase (low cAMP-PK) yielded a phosphoprotein on SDS–PAGE with an apparent molecular weight of 26K (Figure 1a, lane 3), whereas phosphorylation in the presence of 6 $\mu$M calmodulin and 1.5 $\mu$M free $Ca^{2+}$ yielded a phosphorylated product of $M_r$ 28K (Figure 1a, lane 2). This was in agreement with previous findings made in this laboratory (Chiesi et al., 1983). The apparent molecular weights were determined by calibration of the gels with different mixtures of molecular weight marker proteins (see Materials and Methods). It must be kept in mind, however, that the apparent molecular weights of phosphorylated PLB may vary considerably depending on the gel type, the gel system, and the percentage of cross-linker in the gel (unpublished results in this laboratory; Inui et al., 1985).

If the phosphorylation reaction was performed in the presence of higher amounts of the catalytic subunit of cAMP-dependent protein kinase (600 units/mL, high cAMP-PK), the resulting phosphoprotein shifted to higher apparent molecular weight, and a concomitant increase in the radioactivity incorporated was observed. To clarify the ob-

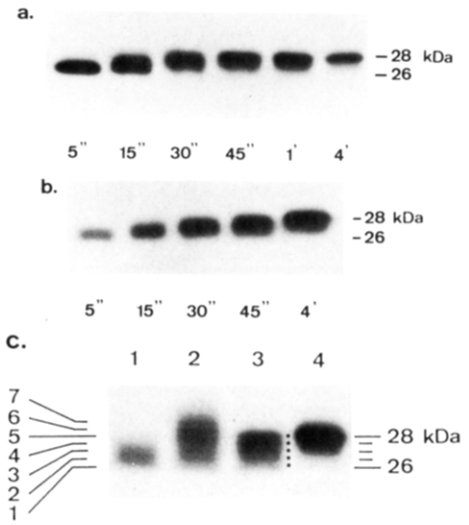


FIGURE 2: (a) ATP/[$\gamma$-$^{32}$P]ATP chase phosphorylation of phospholamban. Fifty micrograms of SR vesicles was incubated in the phosphorylation medium in the presence of 600 units/mL cAMP-PK. The phosphorylation reaction was started by a pulse of 100 nM [$\gamma$-$^{32}$P]ATP at a specific activity of 0.5 $\mu$Ci/nmol. The first aliquot was withdrawn after 5 s and stopped with SDS sample buffer as described for Figure 1a. At 10-s reaction time, cold ATP was added to a final concentration of 300 $\mu$M. Further aliquots were withdrawn at the times indicated. The samples were analyzed on a 15% SDS gel and autoradiographed (see Materials and Methods for details). (b) Time course of cAMP-PK-dependent phosphorylation of phospholamban. Fifty micrograms of SR was incubated as in (a). The phosphorylation reaction was started by adding 300 $\mu$M [$\gamma$-$^{32}$P]ATP at a specific activity of 0.1 $\mu$Ci/mol, and aliquots were withdrawn at the times indicated and treated as indicated in Figure 1a. Autoradiography conditions are described under Materials and Methods. (c) Analysis of PLB phosphorylation products on high-resolution SDS–PAGE. Fifteen micrograms of SR vesicles was phosphorylated under standard conditions in the presence of no addition (lane 1, control), 6 $\mu$M calmodulin (lane 2), 90 units/mL cAMP-PK (lane 3), and 600 units/mL cAMP-PK (lane 4). After 4 min, the phosphorylation was stopped as described for Figure 1a, and the samples were run on a 15% high-resolution SDS gel and autoradiographed (see Materials and Methods).

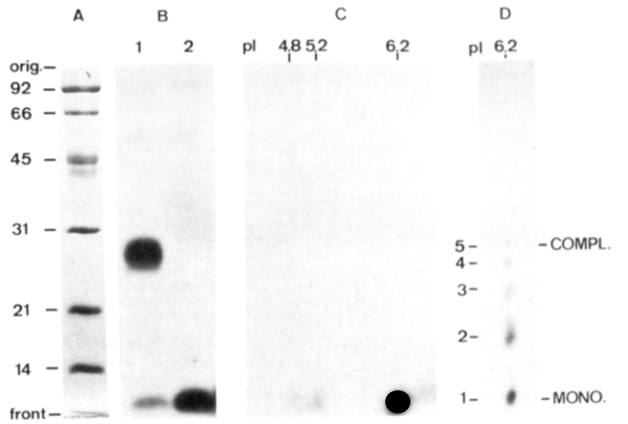


FIGURE 3: Relative position of the phosphorylated PLB complex and of the phosphorylated PLB monomer on a 12% SDS gel. (A) Molecular weight markers (Bio-Rad), Coomassie blue stained. (B) Thirty micrograms of SR vesicles was phosphorylated in the presence of 600 units/mL cAMP-PK (high cat) under standard conditions (see Materials and Methods). The sample of lane 1 was incubated 10 min at room temperature in the presence of 2.5% SDS, whereas the sample of lane 2 was boiled for 5 min in the presence of 2.5% SDS prior to its application to the gel. Panels A and B derive from the same gel. (C) Thirty micrograms of SR vesicles was phosphorylated under identical conditions as described in (B) and processed for analysis on an IEF gel (see Materials and Methods) in the first dimension. After completion of focusing, the strip was cut out from the IEF gel, incubated for 20 min in 10 mL of equilibration buffer (see Materials and Methods), and then analyzed in the second dimension on an identical 12% SDS gel as used for (A) and (B). (D) SR vesicles were phosphorylated as described in (C). After IEF, the band with p*I* 6.2 was cut out, fixed for 30 min in 10% w/v TCA and 30% v/v ethanol followed by an overnight wash in 5% TCA and 30% ethanol, and then analyzed in a second dimension on 12% SDS–PAGE.

servation, it was decided to investigate in detail (i) the time dependency of PLB phosphorylation in the presence of high cAMP-PK and (ii) the gel migration properties of pulse-labeled PLB chased with high cAMP-PK and cold ATP. Figure 2 analyzes this shift in molecular weight upon phosphorylation of PLB by high cAMP-PK. The apparent molecular weight increased stepwise with time in parallel with the increase in the amount of incorporated radioactivity (Figure 2b), whereas in the pulse–chase experiment (Figure 2a), the same shift was observed but with approximately constant amounts of incorporated radioactivity. The slight decrease in the amount of radioactivity at longer reaction times was probably due to the partial dephosphorylation of PLB by endogenous phosphatases. These results are consistent with the proposal that the PLB complex contains several sites which are sequentially phosphorylated by the cAMP-PK (Wegener & Jones, 1984). Within 4 min of phosphorylation time, PLB was maximally phosphorylated and its position on the gel shifted to its highest apparent molecular weight (28K). Using high-resolution SDS–PAGE (see Materials and Methods), it was possible to detect five distinct phosphorylated bands, ranging in molecular weight from 26K to 28K when PLB was phosphorylated under the standard phosphorylation conditions described (Figure 2c, lane 3, bands 1–5). This corresponds to the recent data by Imagawa et al. (1986), who have interpreted the molecular weight shift (Wegener & Jones, 1984) and the coexistence of five distinct phosphorylated bands in the case of cAMP-PK-dependent phosphorylation with the proposal that PLB is a pentamer of five identical 5-kDa parts. Phosphorylation for 4 min in the presence of 600 units/mL cAMP-PK yielded predominantly band 5 (apparent $M_r$ 28K, Figure 2c, lane 4), suggesting again that under these conditions PLB was maximally phosphorylated.

In principle, however, one cannot exclude the alternative possibility that only one subunit of PLB is phosphorylated at five different sites. To distinguish between these two possibilities, a flatbed isoelectric focusing system in agarose gels has been developed which allows the separation of PLB species after phosphorylation according to their apparent p*I* value. An interesting feature of this IEF system is that PLB becomes fully dissociated into its subunits. To illustrate this important point, phosphorylated PLB species were first separated on the IEF system and then analyzed in a second dimension on a SDS–PAGE. Figure 3C shows that all major phosphorylated bands visualized on the IEF gel migrated to a position corresponding to that of the PLB monomer (see Figure 3B for the relative positions of monomers and PLB complex on SDS–PAGE). Elution of ampholines and detergents from the IEF strip prior to application on the SDS–PAGE system by washing them overnight in 5% TCA and 33% ethanol induces reassociation of the PLB subunits. Under conditions of partial elution, it is possible to obtain partial reassociation so that all possible intermediates between monomer and fully oligomerized PLB become apparent (Figure 3D). This interesting observation also clearly demonstrates that the PLB complex is composed of five subunits.

SR vesicles were phosphorylated under various conditions, and the characteristics of the PLB subunits were then analyzed by IEF. Phosphorylation of PLB by cAMP-PK or Calm-PK yielded two products with distinct p*I* values (Figure 4). In the case of the cAMP-PK-dependent phosphorylation, the p*I*

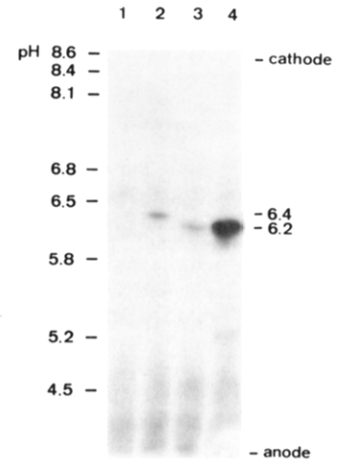


FIGURE 4: Autoradiography of phosphorylated SR vesicles analyzed on an agarose IEF gel. Ten micrograms of SR was phosphorylated under standard conditions in the presence of no addition (lane 1, control), 6 $\mu$M calmodulin (lane 2), 60 units/mL cAMP-PK (lane 3), and 600 units/mL cAMP-PK (lane 4). The phosphorylation reactions were terminated after 4 min, and the samples were processed for IEF analysis (2% pH 3.5–10 carrier ampholine) and autoradiographed as described under Materials and Methods.

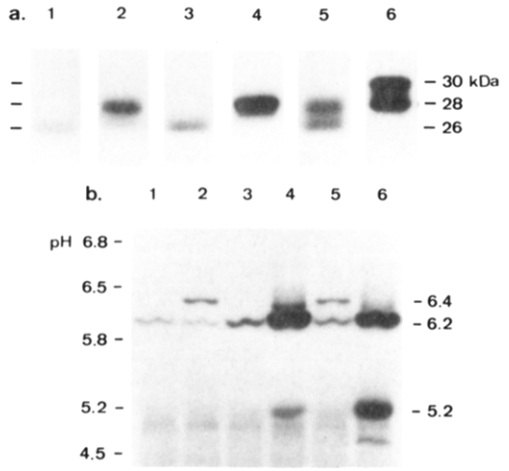


FIGURE 5: Concerted phosphorylation of PLB by Calm- and cAMP-dependent kinases. (a) SDS–PAGE analysis. The phosphorylations were carried out for 4 min in the presence of the following: no addition (lane 1, control), 6 $\mu$M calmodulin (lane 2), 60 units/mL cAMP-PK (lane 3), 600 units/mL cAMP-PK (lane 4), 6 $\mu$M calmodulin plus 60 units/mL cAMP-PK (lane 5), and 6 $\mu$M calmodulin plus 600 units/mL cAMP-PK (lane 6). Each lane contained 30 $\mu$g of SR protein. (b) IEF analysis. Phosphorylated SR vesicles were separated on an agarose IEF gel containing a 1:1 mixture of pH 3.5–10 and pH 5–8 carrier ampholines. This type of IEF gel affords maximum separation of the phosphorylation products at pH 6.2 and 6.4. The samples were as in (a). The phosphorylation conditions, the stopping of the reaction, and the conditions for PAGE and IEF are described in the previous legends and under Materials and Methods.

observed was 6.2 while phosphorylation in the presence of calmodulin–$Ca^{2+}$ yielded a band with p*I* 6.4. Phosphorylation with low cAMP-PK (60 units/mL) yielded an amount of phosphoprotein at p*I* 6.2 approximately equivalent to that produced with Calm-PK at p*I* 6.4. When the concentration of cAMP-PK was increased from 60 units/mL (low cAMP-PK) to 600 units/mL (high cAMP-PK), the p*I* of the phosphorylated product did not shift, but a marked increase in the amount of radioactivity was detected (see above). These data demonstrate that in the case of high cAMP-PK-dependent phosphorylation, multiple phosphorylation of the same subunit apparently does not occur, more subunits per PLB complex instead being phosphorylated.

*Concerted Phosphorylation of Phospholamban by cAMP-PK and Calm-PK.* Phosphorylation of SR vesicles in the presence of both 6 $\mu$M calmodulin (Calm-PK) and 60 units/mL cAMP-PK (low cAMP-PK) yielded two distinct bands on SDS–PAGE (Figure 5a, lane 5) as previously reported (Chiesi et al., 1983). The apparent molecular weight and the intensity of these two bands corresponded fairly well to those observed when the phosphorylation was carried out with Calm-PK or cAMP-PK alone. This indicates that under the experimental conditions a double phosphorylation of the same PLB complex by the two kinases did not occur. On the other hand, when the phosphorylation reaction was performed in the presence of high cAMP-PK (600 units/mL) and 6 $\mu$M calmodulin (Calm-PK), a new band appeared at the apparent molecular weight of 30K (Figure 5a, lane 6). Again, using high-resolution SDS–PAGE, we could detect five intermediate bands, ranging in molecular weight from 28K to 30K, in agreement with the findings by Imagawa et al. (1986). However, under the experimental conditions used here (see Materials and Methods), the 28- and 30-kDa bands [corresponding to bands 5 and 10 of Imagawa et al. (1986)] were clearly predominant, while the intermediate bands were hardly detectable (data not shown). Figure 5b shows IEF gels of samples analogous to those of the SDS–PAGE experiments of Figure 5a. In agreement with the observations obtained with SDS–PAGE, low cAMP-PK and Calm-PK did not induce double phosphorylation of the same PLB subunit (Figure 5b, lane 5). On the other hand, upon phosphorylation with both high cAMP-PK and high Calm-PK, the p*I* 6.4 band, i.e., the product of the Calm-PK-dependent phosphorylation, completely disappeared, whereas the p*I* 6.2 phosphoprotein, i.e., the product of the cAMP-PK-dependent phosphorylation, was still present. An additional and prominent band was now detected at p*I* 5.2 (Figure 5b, lane 6). The acidic shift is conveniently explained with the incorporation of a second phosphate group into the PLB subunit, originally displaying p*I* 6.4. The increasing apparent molecular weight in SDS–PAGE of the corresponding PLB complex phosphorylated under these conditions is compatible with the proposal of doubly phosphorylated PLB subunits. In the presence of high cAMP-PK (600 units/mL), other minor bands were detected on IEF gels (Figure 5b, lane 4, p*I* 5.2, and lane 6, p*I* 4.8). Analysis using two-dimensional gels demonstrated that these minor components also represent PLB monomers (see also Figure 3). It is likely that at these extremely high concentrations the cAMP-PK phosphorylates an additional hitherto undetected phosphorylation site. However, these additional phosphorylation products represent only a minor percent of the total PLB population and were therefore not further considered.

*Semiquantitative Analysis of the Products Phosphorylated under Different Conditions.* In performing experiments of the type presented in Figure 5, it was consistently observed that when high cAMP-PK and calmodulin were added at the same time, the amount of radioactivity associated with PLB was consistently higher than what was expected from the sum of the single phosphorylation levels. A semiquantitative analysis (see Materials and Methods) of the phosphorylation products obtained under various conditions was thus carried out. The results of Table I show that while in the presence of calmodulin and low cAMP-PK the amount of radioactivity incorporated in PLB was approximately equal to the sum of two independent phosphorylation levels, in the presence of high cAMP-PK and calmodulin, excess incorporation took place. Two possible explanations were considered. (i) The two kinases influence each other when present at the same time. (ii) A portion of

Table I: Phosphorylation of Cardiac SR Vesicles under Various Conditions:[a] Semiquantitative Analysis of the Radioactivity Incorporated into the $M_r$ 26K–30K Phospholamban Complex[b]

| SR prepn | 6 μM calmodulin | 60 units/mL cat[c] | 600 units/mL cat[c] | 60 units/mL cat[c] + 6 μM calmodulin | 600 units/mL cat[c] + 6 μM calmodulin |
|---|---|---|---|---|---|
| 1 | 17 | 9 | 95 | 28 | 146 |
| 2 | 30 | 28 | 78 | 58 | 138 |
| 3 | 8 | 5 | 93 | 17 | 126 |
| 4 | 28 | 28 | 91 | 52 | 156 |

[a] Each sample contained 30 μg of SR protein, which was phosphorylated under the conditions described under Materials and Methods. The samples were separated on a 15% Laemmli gel and autoradiographed, and the labeled phosphoproteins in the molecular weight range 26K–30K were cut out from the gel and counted in a liquid scintillation counter. [b] The results represent relative units, normalized for the amount of radioactivity incorporated per milligram of SR protein in the case of high cAMP-PK dependent phosphorylation. [c] cat = catalytic subunit of the cAMP-dependent protein kinase.

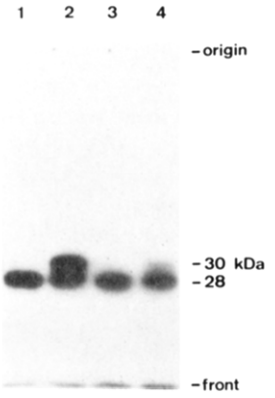


FIGURE 6: Autoradiography of sequentially phosphorylated PLB analyzed on a 12% SDS gel. Thirty micrograms of SR protein was prephosphorylated under standard conditions in the presence (lane 2) or absence (lane 1) of 6 μM calmodulin and 1 mM cold ATP for 4 min. The reaction was quenched as described under Materials and Methods, and the resuspended vesicles were chase phosphorylated in the presence of 300 μM [$\gamma$-$^{32}$P]ATP and 600 units/mL cAMP-PK. Lanes 3 and 4 show the reversed experiment, i.e., prephosphorylation carried out in the presence (lane 4) or absence (lane 3) of 600 units/mL cAMP-PK. After the vesicles were washed and resuspended, they were chase phosphorylated in the presence of 6 μM calmodulin and 300 μM [$\gamma$-$^{32}$P]ATP. The stopping of the reaction and the conditions for PAGE and autoradiography are described in the previous legends and under Materials and Methods.

the substrates available (PLB subunits) is not recognized by one of the kinases unless previously phosphorylated by the other. To discriminate between these two possibilities, chase phosphorylation experiments were carried out in which sequential phosphorylation by the two kinases was allowed but with only one of them present at each time. Figure 6 shows the experiment in which nonphosphorylated PLB (lane 1) or PLB prephosphorylated in the presence of calmodulin and cold ATP (lane 2) was subsequently chase phosphorylated in the presence of high cAMP-PK and labeled ATP. Quantitation of the phosphorylation products revealed that when the SR membranes were prephosphorylated in the presence of calmodulin, 37% ± 8% (mean of four different experiments) additional sites became available to phosphorylation by the cAMP-PK. Control experiments ensured that the second treatment did not cause artifacts due to partial dephosphorylation of PLB or to incompletely removed calmodulin (data not shown). Figure 6 (lanes 3 and 4) shows the opposite experiment, i.e., prephosphorylation of PLB in the presence of high cAMP-PK and cold ATP, followed by chase phosphorylation in the presence of calmodulin and labeled ATP. In this case, no significant difference in the amount of radioactivity incorporated could be observed between the prephosphorylated and the nonphosphorylated membranes. In lane 2, the 30-kDa product was clearly visible, revealing a PLB complex containing doubly phosphorylated subunits, deriving from subunits phosphorylated by Calm-PK during the prephosphorylation step (Figure 6, lane 2). On the other hand, when the sequence of phosphorylation by the two kinases was reversed, the 30-kDa band became much less evident, even if not compeltely absent (Figure 6, lane 4). This indicates that a portion of PLB cannot be phosphorylated by cAMP-PK unless it is prephosphorylated by Calm-PK. If this happens, double phosphorylation of the same PLB subunit occurs (see Figure 5a and lane 6 of Figure 5b). It is noteworthy to mention that this double phosphorylation of the same PLB subunit was observed only when cAMP-PK was present at high concentrations (600 units/mL) but not when present at low concentrations (60 units/mL).

*Stimulation of the Initial Rate of $Ca^{2+}$ Uptake by Phosphorylated Phospholamban.* Several investigators have observed a stimulation of the initial rate of $Ca^{2+}$ uptake by cardiac SR membranes after phosphorylation of PLB by the cAMP-dependent kinase (Wray et al., 1973; Kirchberger et al., 1974) and by the calmodulin-dependent kinase (Katz & Remtulla, 1978; Le Peuch et al., 1979). The stimulatory effects of the cAMP-PK- and Calm-PK-dependent phosphorylations of PLB have been shown to be additive (Kranias et al., 1980; Tada et al., 1983; Chamberlain et al., 1983). Experiments were performed to investigate whether increasing amounts of phosphorylation by cAMP-PK, yielding more phosphorylated PLB subunits per complex, resulted in augmented stimulation of the uptake of $Ca^{2+}$. The initial rate of $Ca^{2+}$ uptake at 37 °C and 0.5 μM free $Ca^{2+}$ (0.116 ± 0.015 μmol $mg^{-1}$ $min^{-1}$, mean of three SR preparations) was stimulated 2.1 ± 0.1 times, 1.9 ± 0.15 times, and 3.3 ± 0.8 times by low cAMP-PK (60 units/mL), 6 μM calmodulin, and low cAMP-PK *and* calmodulin, respectively. Interestingly, if the cAMP-PK in the medium was increased to 600 units/mL, a much stronger stimulation of the initial $Ca^{2+}$ uptake rate was obtained (4.2–4.8 times). Although the additional stimulation by calmodulin was smaller than in the case of low cAMP-PK, a clear increase in the initial rate of $Ca^{2+}$ uptake was still evident.

## Discussion

The subunit composition of PLB is a controversial issue. Different laboratories have proposed PLB to be a dimer of two identical subunits with an apparent molecular weight of 11K (Le Peuch et al., 1979; 1980; Lamers & Stinis, 1980; Tada et al., 1983), a heterotrimer (Louis et al., 1982), or a homotetramer (Kirchberger et al., 1982). On the basis of amino acid composition data, a molecular weight of 5.5K has been postulated for the monomer, without actually detecting it directly on SDS–PAGE gels (Le Peuch et al., 1980; Capony et al., 1983). It has now gradually become evident that PLB could indeed be a pentamer composed of five similar subunits of molecular weight around 5K (Jones et al., 1985; Inui et al., 1985). However, the molecular weight determinations of the putative monomer of PLB have all been performed on SDS–

PAGE gels, which, particularly in this molecular weight range, may lead to molecular weight miscalculation: The observed molecular weight of several proteins has indeed been found to vary significantly depending on the gel type, the gel concentration, the percentage of cross-linker in the gel, and the phosphorylation state of the protein. In this work, the gel systems have been very carefully calibrated, and it has been found that in the molecular weight range below about 15K, the correlation between mobility and molecular weight of proteins is very poor. Therefore, the 11K band frequently observed upon "monomerization" of the PLB might in fact correspond to the 5K monomer of phospholamban. In addition, it is now well-known that the phosphorylation of PLB results in the change of its mobility on SDS–PAGE gels (Wegener & Jones, 1984; Imagawa et al., 1986), probably due to a conformational change as already observed for several other proteins (Hofmann et al., 1975; Ahmad et al., 1982; Stadel et al., 1983; Creamer et al., 1984). Thus, the interpretation of the behavior of PLB in SDS–PAGE is bound to be difficult and frayed with uncertainties. In the present work, additional information has been obtained by means of a special IEF procedure, which allows the separation of highly hydrophobic membrane proteins, including PLB. Since radioactively labeled PLB could be detected at very high resolution using material derived directly from solubilized SR membranes, the identification of the phosphorylation products of the endogenous, calmodulin-dependent protein kinase became easily possible. Indeed, on IEF gels the incorporation of a $P_i$ group into a protein is expected to induce a shift in p*I* which could be much more evident than the mobility change observed on SDS–PAGE. In addition, it has been found that treatment with the mixture of 2% Triton, 1% Chaps, and 1% carrier ampholines used in the IEF system described in this work completely dissociated the PLB complex into its subunits. It is appropriate to mention at this point that the disruption of PLB into its subunit components could only be achieved in previous studies under denaturating conditions, i.e., by using SDS or Triton or by boiling the SR membranes rating conditions, i.e., by using SDS or Triton or by boiling the SR membranes in the presence of SDS. That under the experimental conditions used here the monomer subunits of PLB in their different states of phosphorylation were indded visualized was established by several indirect controls: (i) No p*I* differences was observed between PLB phosphorylated in the presence of low or high cAMP-PK (see Figure 4, lanes 3 and 4). Under these conditions, the only difference was the amount of the label incorporated in the band at p*I* 6.2, indicating that the number of phosphorylated PLB subunits increased with the amount of cAMP-PK. If the p*I* 6.2 band were representative of intact PLB complexes, then it would have shifted toward lower p*I* values upon incorporation of additional $P_i$ groups. (ii) On two-dimensional SDS–PAGE, the three major bands visible on IEF gels migrated at a position corresponding to an apparent molecular weight of 11K (see Figure 3). In addition, one-dimensional SDS–PAGE using Triton, Chaps, and ampholines in the sample buffer produced the same pattern as the standard samples, previously boiled for 5 min in the presence of 2.5% SDS; i.e., the phosphorylated PLB migrated at the apparent molecular weight position corresponding to 11K (see Figure 3). As discussed above, this band is likely to correspond to the 5000-Da monomer postulated by others (Le Peuch et al., 1980; Capony et al., 1983). Indeed, when the detergents and the carrier ampholines were washed out from the agarose IEF gel strip prior to the second dimension on SDS–PAGE, five distinct phosphorylated spots, ranging in molecular weight between 11K and 26K, were detected (see Figure 3). Evidently, under these conditions, the fully dissociated PLB subunits reassociated partially, yielding the dimeric, trimeric, tetrameric, and pentameric configurations. If it is assumed that PLB bis composed of six nearly identical subunits, their molecular weights should be about 5000, even though in the SDS–PAGE system used in this work the apparent molecular weight is about 11 000. All these observations strongly support the conclusion that the bands at p*I* 6.2 and 6.4 on IEF gels represent PLB subunits, phosphorylated by cAMP-PK and Calm-PK, respectively, and not the PLB complex. The minor difference in the p*I* may be due to two different phosphorylation sites for the two kinases on the same PLB subunit or, alternatively, to a minor difference between the two subunits.

*cAMP-Dependent Phosphorylation of PLB.* The time dependency and the pulse–chase experiments shown in Figure 2a,b have provided support for the findings (Wegener & Jones, 1984), indicating a sequential insertion of $P_i$ groups into the PLB complex phosphorylated by the cAMP-PK. In agreement with a more recent report (Imagawa et al., 1986), under appropriate conditions (see Figure 2c, lane 3) five distinct phosphorylated bands ranging in apparent molecular weight between 26K and 28K could be detected. These bands corresponded to PLB complexes phosphorylated to five different levels. In the presence of high concentrations of cAMP-PK, most of the radioactive label was concentrated in the band corresponding to position 5 (see Figure 2c, lane 4), indicating that all available PLB complexes were maximally phosphorylated (i.e., probably contained five $P_i$ groups). These findings and the analysis of PLB subunits on IEF gels (see Figures 3–5) strongly support the concept of five very similar (if not identical) subunits, each of them phosphorylated at one site only, and rule out the possibility of a special subunit in the PLB complex with multiple phosphorylation sites for cAMP-PK.

The incubation of SR membranes in the presence of 60 units/mL cAMP-PK resulted in the phosphorylation of one to two subunits per PLB complex and in the 2-fold stimulation of the $Ca^{2+}$ uptake rate, as normally reported in the literature. The observations that the stimulation of $Ca^{2+}$ uptake increases in parallel with the increasing phosphorylation level and that the complete phosphorylation of the five PLB subunits produces a 400–500% stimulation of the rate of $Ca^{2+}$ translocation are worth emphasizing.

*Calmodulin-Dependent Phosphorylation of PLB.* The endogenous calmodulin-dependent kinase also induces multiple phosphorylation of the same PLB complex. Imagawa et al. (1986) have suggested 2 independent sites on each PLB monomer for Calm-PK, based on the observation of 10 distinct SDS–PAGE bands, ranging in molecular weight between 24K and 28K. In this study, a similar band pattern could be observed only in the presence of calmodulin and low concentrations of cAMP-PK, thus indicating that the SR preparation used by Imagawa et al. was probably contaminated by endogenous cAMP-PK. In the presence of calmodulin alone, five to eight bands were detected, depending on the SR preparation, ranging in molecular weight between 26K and 28K on SDS–PAGE (Figure 2c, lane 2). However, only the five bands with apparent molecular weights between 27K and 28K resulted from calmodulin-dependent phosphorylation. The bands with lower apparent molecular weights (26K–27K) were also observed in the control phosphorylation experiments in the absence of exogenous calmodulin (Figure 2c, lane 1). The p*I* value of 6.2 after IEF analysis of these background phosphorylation products (Figure 5b,s lane 1) demonstrates

that they did not arise from endogenous calmodulin-dependent kinase activity but rather from endogenous cAMP-PK. In analogy with the situation obtained with cAMP-PK, our combined results from SDS–PAGE and IEF gels indicate that the endogenous Calm-PK phosphorylated a single site on each of the five subunits of PLB.

The maximal levels of phosphorylation in the presence of calmodulin were consistently lower than those obtained with high concentrations of cAMP-PK, indicating that only a fraction of the total PLB population was available to the endogenous Calm-PK. Experiments in progress indicate that the Calm-dependent phosphorylation may be concentrated in the cisternal compartments of the SR system, while the PLB distribution closely follows that of the Ca-ATPase. Indeed, a detailed study using various subfractions obtained from the crude cardiac SR membranes has shown that both the calmodulin-dependent phosphorylation of PLB and the concomitant stimulation of $Ca^{2+}$ uptake are restricted to the membranes originating from the cisternae compartments (unpublished results).

*Concerted Phosphorylation of PLB by cAMP-PK and Calm-PK.* In the presence of 60 units/mL cAMP-PK and 6 $\mu$M calmodulin, the phosphorylation level of PLB (see Table I) as well as the stimulation of $Ca^{2+}$ uptake was clearly additive. It is now generally accepted that under these conditions each PLB subunit is phosphorylated at two distinct sites by the two kinases. The combined IEF and SDS–PAGE analysis (Figure 5a,b) has demonstrated, however, not only that different PLB subunits were involved but also that different populations of PLB complexes were phosphorylated by the two kinases. This is in line with previous observations in this laboratory (Chiesi et al., 1983) and indicates that different populations of ATPases may be responsive to the calmodulin- or cAMP-dependent phosphorylations.

On the other hand, when SR vesicles were phosphorylated simultaneously in the presence of calmodulin and high cAMP-PK (600 units/mL), a new set of phosphorylation sites on PLB became apparently available for phosphorylation. Thus, the population of PLB subunits which are usually the substrates for the endogenous Calm-PK was apparently not available for phosphorylation by cAMP-PK but became available to high concentrations of cAMP-PK after phosphorylation by the endogenous Calm-PK. IEF analysis has shown that under these conditions the PLB subunit having a p*I* of 6.4 underwent an acidic shift to p*I* 5.2 when phosphorylated in the presence of calmodulin and high amounts of cAMP-PK. The excess phosphorylation observed in the presence of both calmodulin and high cAMP-PK corresponded approximately to the level of phosphorylation obtained with calmodulin alone. The results presented here help explain the report of Katz and Remtulla (1978) in which the $Ca^{2+}$ pumping of particular SR preparations could be stimulated by cAMP only in the presence of calmodulin.

In conclusion, the present findings support previous results from this laboratory indicating the existence of two distinct populations of PLB complexes which react differently to Calm-PK and cAMP-PK. The differential behavior may be due to small differences in either the hydrophobic microenvironment or the amino acid composition of the subunits of the PLB complexes. Alternatively, and possibly more likely, the inhomogeneous distribution of the kinases and steric hindrance effects could be responsible for the apparent selectivity. The membrane-bound Calm-PK would only phosphorylate the specific population of PLB with which it can interact (localized mainly in the terminal cisternae). The soluble cAMP-PK could in principle reach all PLB molecules but would not be able to recognize its phosphorylation sites in the population of PLB associated with endogenous Calm-PK. Phosphorylation of this population by the endogenous calmodulin-dependent kinase would somehow unmask the other set of sites and render them susceptible to the action of cAMP-PK. However, since these prephosphorylated PLB subunits are a poor substrate for cAMP-PK, the extra set of sites can only be phosphorylated by cAMP-PK when present at high concentrations. Lastly, a comment is in order on the observation that the maximal phosphorylation of PLB induced up to a 5-fold stimulation of the $Ca^{2+}$ uptake rate of cardiac SR vesicles. In the in vitro system used here, this required very high amounts of exogenous cAMP-dependent kinase. Whether the amounts of activated kinases available in vivo are adequate to produce the phosphorylation levels that would activate the SR pumping rate 5-fold is impossible to decide. It is, however, of great importance to establish that the $Ca^{2+}$-ATPase of SR in cardiac cells is potentially able to remove $Ca^{2+}$ from the cytosol at a rate which is much faster than under normal nonstimulated conditions and, in general, much faster than previously thought.

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**Registry No.** PK, 9026-43-1; ATPase, 9000-83-3; Ca, 7440-70-2.

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# Rapid Filtration Study of the Phosphorylation-Dependent Dissociation of Calcium from Transport Sites of Purified Sarcoplasmic Reticulum ATPase and ATP Modulation of the Catalytic Cycle†

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ABSTRACT: Time-resolved filtration measurements using radioactive calcium were conducted to investigate with leaky preparations the kinetic features of the dissociation of transported calcium to the luminal side of the sarcoplasmic reticulum calcium pump, which occurs concomitantly with isomerization of the phosphorylated ATPase. At pH 6 and 20 °C, $Ca^{2+}$ dissociation was moderately fast in the absence of potassium (3–5 $s^{-1}$ at 0.05 mM ATP), implying that the dephosphorylation step (about 1.5 $s^{-1}$) was the main contributor to rate limitation under these conditions. Potassium slowed down $Ca^{2+}$ release but stimulated dephosphorylation, so that in its presence $Ca^{2+}$-releasing isomerization did contribute to rate limitation, especially at neutral pH. At pH 6 in the absence of potassium and in the presence of magnesium, millimolar concentrations of ATP doubled the rate of $Ca^{2+}$ dissociation, as also shown by dual-wavelength detection of fast changes in the absorbance of the $Ca^{2+}$-sensitive dye Antipyrylazo III. Under the same conditions, low-affinity binding of ATP to phosphoenzyme was demonstrated. It is suggested that this low-affinity acceleration by ATP of the crucial step leading to dissociation of transported $Ca^{2+}$ is the specific interaction responsible for the low-affinity acceleration of overall ATPase activity generally observed in the presence of potassium at neutral pH. Hydrolysis of the $Ca^{2+}$-deprived phosphoenzyme was accelerated by ATP in the absence but not in the presence of $Mg^{2+}$ in the dephosphorylation medium. We suggest that metal-free ATP is a more potent activator than Mg·ATP for transitions involving phosphoenzyme.

The membranous sarcoplasmic reticulum ATPase[1] catalyzes ATP-dependent active transport of calcium from the cytoplasm to the reticular lumen. When the catalytic cycle is initiated by adding Mg·ATP to $Ca^{2+}$-saturated ATPase, phosphoenzyme is formed at a fast rate; thereafter, a conformational transition of the phosphorylated ATPase occurs together with diminution of the affinity for calcium of the transport sites, and the bound calcium ions dissociate to the lumen of the SR vesicles. Later, the covalent acyl–phosphate bond in the phosphoenzyme is hydrolyzed, and the ATPase conformation reverts to the initial one to which cytoplasmic calcium may

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[1] Abbreviations: ATPase, adenosinetriphosphatase; SR, sarcoplasmic reticulum; Mops, 4-morpholinepropanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Tes, 2-[[tris(hydroxymethyl)methyl]-amino]ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; ATP, adenosine triphosphate; PCA, perchloric acid; $k_{obsd}$, observed rate constant; $A_{23187}$, calcimycin; $P_i$, inorganic phosphate; $Me_2SO$, dimethyl sulfoxide.

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