Phospholamban of cardiac sarcoplasmic reticulum consists of two functionally distinct proteolipids

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Phosphorylation of phospholamban by either a cAMP-dependent or a calmodulin-dependent kinase stimulates the Ca^{2+} transporting activity of cardiac sarcoplasmic reticulum membranes. It has now been found that phospholamban consists of 2 distinct proteins; one is the specific substrate for the cAMP-dependent phosphorylation, and the other for the calmodulin-dependent kinase. In spite of functional diversity, the 2 polypeptides share a number of properties. Among them, the proteolipid character, M_r, resistance to trypsinization, and subunit composition.

Sarcoplasmic reticulum *Heart* *Phospholamban* *Calmodulin* *Cyclic AMP* *Phosphorylation*

1. INTRODUCTION

Some of the major mechanical effects of catecholamines on cardiac function can be explained by the cAMP-dependent stimulation of the Ca^{2+}-pumping activity of the sarcoplasmic reticulum (SR) membranes, which promotes the sequestration of higher amounts of Ca^{2+} into the luminal space at an increased rate [1]. The stimulation occurs via the phosphorylation of a 22000 M_r proteolipid, named phospholamban, by a cAMP-dependent kinase [2,3]. The correlation between the phosphorylation level of phospholamban and the stimulation of the ATPase activity is well established and a direct hydrophobic interaction between phospholamban and the ATPase, which are present in the membrane in equimolar amounts, has been postulated [4,5]. The phosphorylation of phospholamban induces changes in the physicochemical characteristics of the molecule. This is reflected in alterations in the hydrophobic microenvironment of the ATPase, thus affecting several steps of the Ca^{2+} transport cycle. Interestingly, it has been observed that phospholamban phosphorylation and the concomitant stimulation of Ca^{2+} transport can be induced also by an endogenous, calmodulin-dependent kinase [4,6]. The calmodulin- and cAMP-dependent stimulations appear to be additive, but several aspects of this concerted regulation are controversial. In particular, it is not clearly established whether:

(i) Calmodulin-dependent phosphorylation of phospholamban increases Ca^{2+} uptake by affecting the turnover rate of the ATPase or by improving the coupling efficiency of the transport reaction;
(ii) The cAMP-dependent regulation is completely independent from the calmodulin-dependent phosphorylation or whether the latter is a prerequisite for the cAMP-induced effects;
(iii) The cAMP- and calmodulin-dependent kinases really produce double phosphorylation of the same molecule at two different sites, as usually proposed.

Here, the last of these 3 aspects has been investigated.

2. MATERIALS AND METHODS

Calf and rat heart SR membranes were isolated essentially as in [4].

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2.1. *Ca^{2+}-dependent acyl-P_i level*

The phosphoenzyme intermediate of the Ca^{2+}-ATPase was formed at 0°C in 100 mM NaCl, 5 mM $MgCl_2$, 20 mM morpholinopropane sulfonic acid (pH 7), Ca-EGTA buffers (50 μM free Ca^{2+}), and 1 mg protein/ml. The reaction was started with 200 μM [γ-^{32}P]ATP and stopped after 10 s in 7% trichloroacetic acid. The denatured protein pellet was washed several times in acid and the radioactivity incorporated was counted in a scintillation mixture. Unspecific ^{32}P incorporation was determined similarly but in the absence of Ca^{2+} in the incubation medium.

2.2. *Calmodulin- and cAMP-dependent phosphorylations*

Cardiac SR vesicles (1 mg/ml) were incubated at 0°C for 10 min in a similar medium to that described above, supplemented with 10 μg oligomycin/ml and 10 μM rotenone. Aliquots were then incubated at room temperature in the presence, when required, of 5 μM calmodulin and/or 2 μM cAMP and 50 μg cAMP-dependent kinase/ml. Reactions were started with 200 μM [γ-^{32}P]ATP. After 2 min, 10 mM EGTA was added to dephosphorylate the Ca^{2+}-dependent acyl-P_i. After an additional 30 s, all reactions were stopped in acid and the remaining alkyl-P_i was determined as above. Alternatively, reactions were stopped at various time intervals directly in SDS-containing sample buffer. Aliquots were then analyzed in 15% SDS–polyacrylamide gels [7]. Electrophoresis was performed at 35 mA with 1.5 × 100 mm gels and stopped 2 h after elution of the dye front. After fixing and drying, the gels were autoradiographed.

3. RESULTS AND DISCUSSION

Calf heart SR preparations can be phosphorylated by an endogenous calmodulin-dependent and/or an exogenous cAMP-dependent kinase. Most of the alkyl-P_i (80–90%) was incorporated into a protein with M_r corresponding to that of phospholamban (fig.1). Under optimal conditions, the maximal level of phosphorylation in the presence of either kinase was similar (0.3–0.5 nmol/mg and 0.4–0.6 nmol/mg with the calmodulin-dependent and cAMP-dependent kinase, respectively) and was comparable to that of active Ca^{2+}-pumping units (0.5–0.8 nmol/mg), as judged from the maximal amount of Ca^{2+}-dependent acyl-P_i intermediate of the ATPase. The stoichiometry between the phosphorylation level of phospholamban and ATPase molecules was therefore close to 1:1, as already observed in dog cardiac SR preparations [4]. This finding has led to the concept that the modulation of the Ca^{2+}-pumping activity might occur via a direct phospholamban–ATPase interaction. It was observed in [4] that the 2 phosphorylations are additive. Therefore, it was concluded that dog heart phospholamban contains 2 distinct sets of sites, which are substrates for the cAMP-dependent and

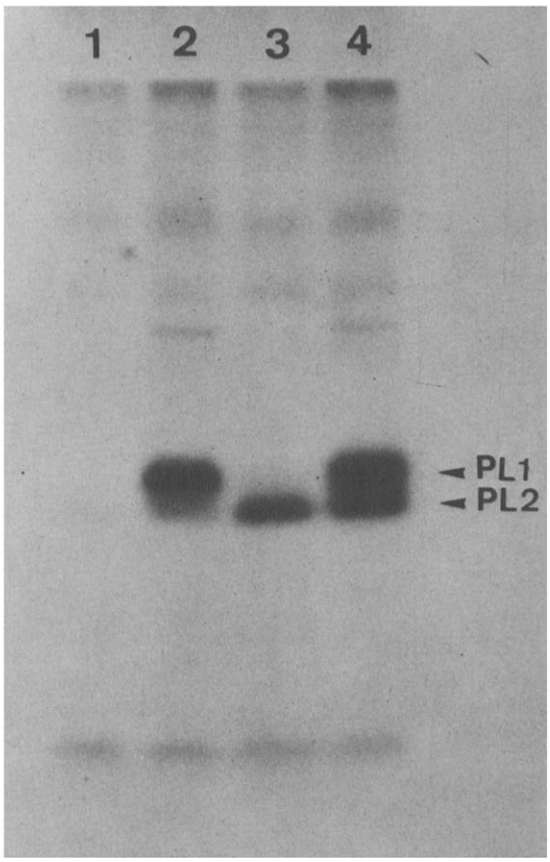

Fig.1. Calmodulin- and cAMP-dependent phosphorylation of calf cardiac SR. SR vesicles were phosphorylated for 2 min as in section 2. The basic reaction mixture was supplemented with: (1) no addition; (2) 5 μM calmodulin; (3) 2 μM cAMP and 50 μg cAMP-dependent kinase/ml; (4) 5 μM calmodulin plus cAMP and cAMP-dependent kinase.

calmodulin-dependent kinases, respectively. Fig.1 shows that also in calf cardiac SR the phosphorylation by the 2 kinases was independent and additive. In addition, the app. M_r of phospholamban when phoshorylated by the cAMP-dependent kinase (PL2 in fig.1, M_r 26000) was different from that after phosphorylation with the calmodulin-dependent kinase (PL1, M_r 28000). Proteins can change their mobility in SDS gels when phosphorylated; therefore, it is possible that a protein, when phosphorylated at 2 different sets of sites, displays a different apparent M_r in gels. However, fig.1 (4) shows that in SR vesicles phosphorylated in the presence of both cAMP- and calmodulin-dependent kinases, a new labelled band was not observed and superimposition of the 2 bands named PL1 and PL2 did not occur. Therefore, double phosphorylation of a same phospholamban subunit is very unlikely, and the results indicate that PL1 and PL2 are 2 distinct polypeptides, one the specific substrate for the cAMP-dependent kinase (PL2), the other for the calmodulin-dependent kinase (PL1). To rule out any artifactual interpretations, the time dependency of the formation of PL1 and PL2 in sequential phosphorylation experiments was investigated. Fig.2 shows that the maximal phosphorylation levels of PL1 and PL2 induced by the subsequent addition of calmodulin- or cAMP-dependent kinase, did not depend on the pre-phosphorylation of PL2 or PL1, respectively. This observation provides a compelling demonstration that PL1 and PL2 are indeed 2 independent and distinct proteins. The presence of 2 functionally different phospholamban species in heart SR has not been observed before. It is in principle possible, that the observation reflects species characteristics of heart SR. SR vesicles were thus prepared from rat hearts and it was found that also in this case cAMP-dependent and calmodulin-dependent kinases induce the formation of 2 different phosphoproteins corresponding to PL2 and PL1 (fig.3). However, in rat labelled protein bands were rather diffuse, so that a consistent overlap occurred. Even though the rat results were not as clear as those obtained with calf heart SR, they still indicate that the presence of 2 different phosphoproteins in the region of phospholamban is not a peculiarity of calf heart SR.

The 25000 M_r protein named phospholamban is composed by strongly associated subunits, of which a 11000 M_r component carries the phosphorylatable sites [4,8]. The dissociation between subunits is induced by boiling the protein in SDS prior to electrophoresis [8,9]. A similar treatment of the phosphorylated calf hart SR proteins led to the complete disappearance of PL1 and PL2 (fig.4), and the appearance of radioactivity in both

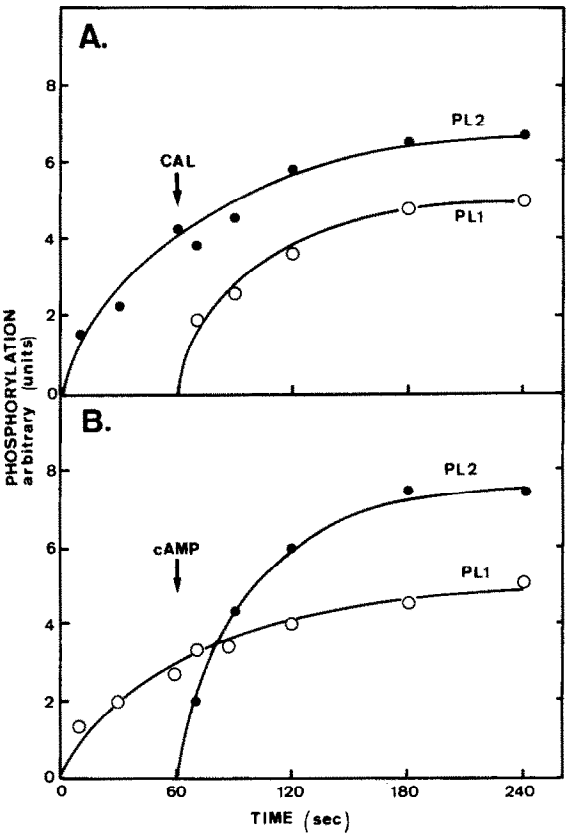

Fig.2. Sequential phosphorylation of calf cardiac SR. (A) SR vesicles were phosphorylated as in section 2 in the presence of 5 μM calmodulin. At the times indicated, aliquots were withdrawn and quenched in SDS-sample buffer. At 1 min an additional 200 μM [γ-^{32}P]ATP was added to the reaction mixture, together with 2 μM cAMP and cAMP-dependent kinase (arrow). (B) The experiment was done as above, but phosphorylation was started in the presence of 2 μM cAMP and cAMP-dependent kinase. After 1 min calmodulin (CAL) was added. The relative levels of phosphorylated PL1 and PL2 formed were analyzed densitometrically on autoradiograms, after separation of the proteins on gels (section 2).

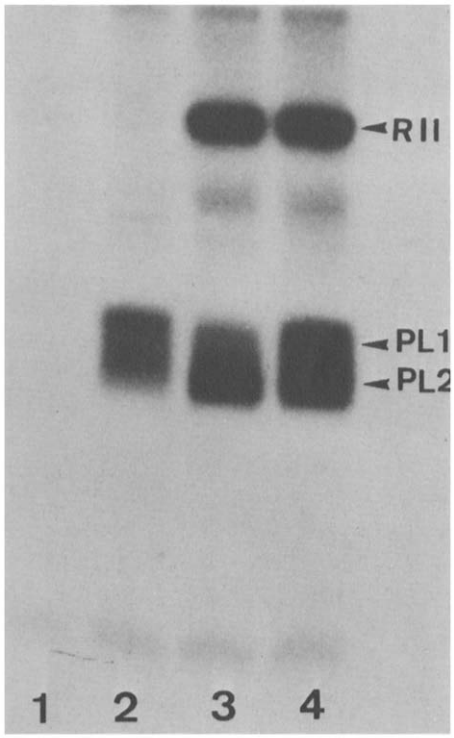

Fig.3. Calmodulin- and cAMP-dependent phosphorylation of rat heart SR. The experiment was performed exactly as in fig.1, but with rat cardiac SR vesicles.

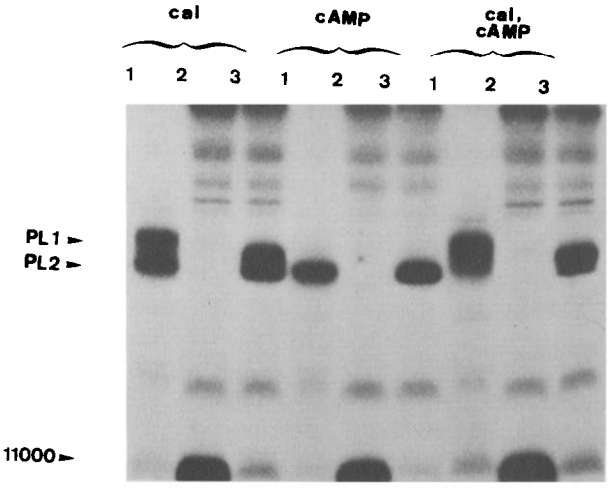

Fig.4. Effect of boiling and trypsinization on PL1 and PL2. Calf cardiac SR vesicles were phosphorylated as in fig.1 in the presence of calmodulin and/or cAMP and cAMP-dependent kinase, as indicated: (1) control phosphorylation pattern; (2) samples in SDS were boiled 4 min before electrophoresis; (3) phosphorylated samples were incubated 5 min with 50 μg trypsin/ml.

cases in a protein of M_r ~11000. Thus, although clearly different, PL1 and PL2 still retain this property of phospholamban. The phosphorylated form of phospholamban is rather resistant to proteolytic digestion by trypsin [10]. Fig.4 shows the results of trypsinization of phosphorylated calf cardiac SR. Even though most of the SR proteins were completely degraded by the treatment (not shown), PL1 and PL2 were only minimally affected. For unknown reasons, the apparent M_r of PL1 increased after trypsinization of the membranes, and the labelled protein band was more diffused.

Another interesting property of phospholamban is its extractability from the membrane into acidified chloroform:methanol mixtures, an observation that led to its classification as an acidic proteolipid [11]. In two separate experiments, calf heart SR vesicles were phosphorylated either with calmodulin or the cAMP-dependent kinase to obtain membranes containing only labelled PL1 or PL2. The vesicles were then washed, lyophylized, extracted with acidified chloroform:methanol, and applied to a HPLC silica column to isolate phospholamban, as in [12]. PL1 and PL2 were quantitatively recovered in the organic phase and it was found that the radioactivity distribution pattern in the fractions eluted from the HPLC columns were identical (fig.5A). The first peak eluted corresponded to phosphorylated lipid components of the membranes. After decreasing the hydrophobicity of the elution medium (arrow) another peak was obtained. The gel electrophoresis experiment of fig.5B shows that the radioactivity corresponded to labelled PL1 and PL2, respectively, indicating that both proteins behaved as acidic proteolipids. However, the apparent M_r difference between PL1 and PL2 was less evident after HPLC. Proteins pre-treated by HPLC may yield more diffused bands on SDS gel electrophoresis, and this might have occurred with PL1 and PL2. However, it is also possible that the HPLC treatment in organic solvents has removed tightly bound lipids from PL1 and PL2 [12], affecting their electrophoretic mobility.

In conclusion, heart SR phospholamban can be resolved into 2 functionally distinct proteolipid

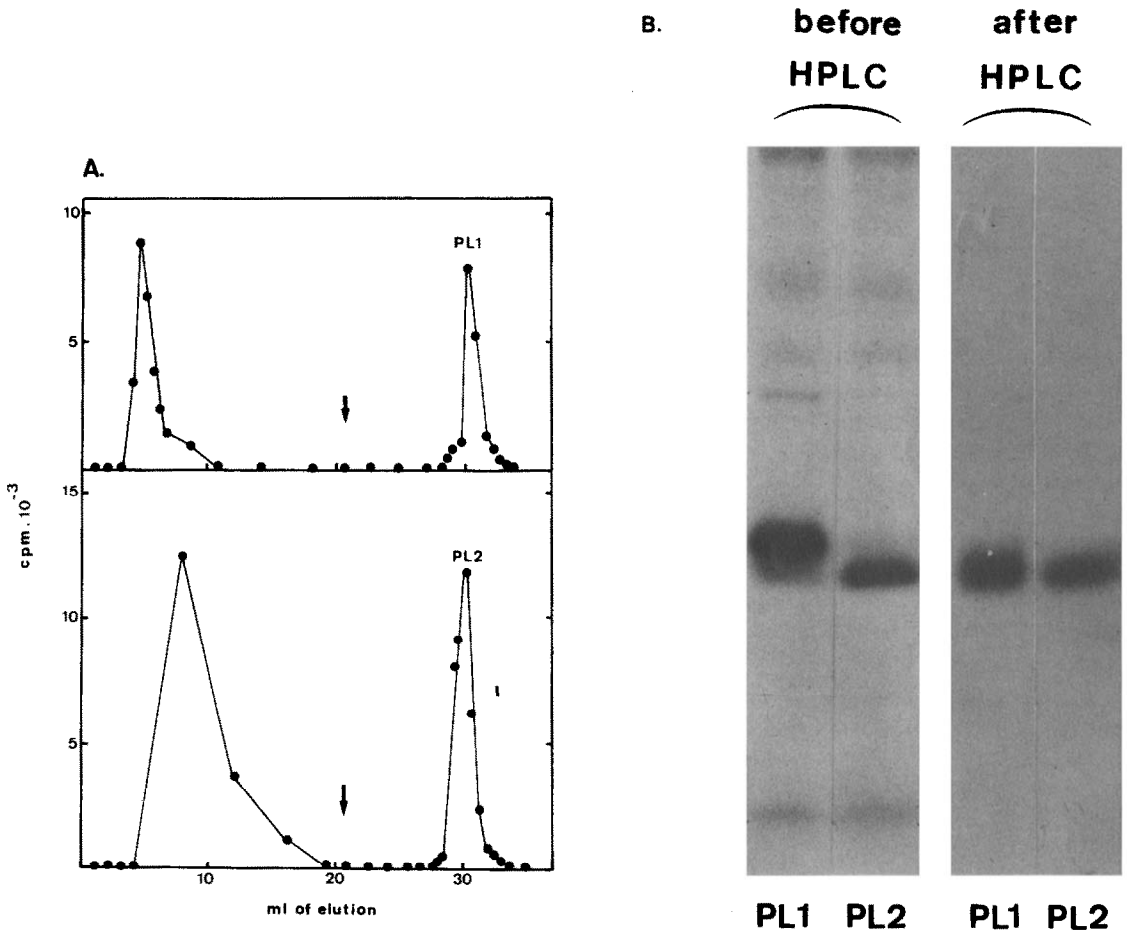

Fig.5. HPLC of organic extracts from calf cardiac SR membranes. SR vesicles were phosphorylated with [γ-^{32}P]ATP in the presence of either calmodulin or cAMP and cAMP-dependent kinase, to obtain labelled PL1 or PL2, respectively. Acidic proteolipids were then extracted in organic solvents and applied to a HPLC silica column as in [12]. The column was eluted first with chloroform:methanol:H_2O:trifluoroacetic acid (200:100:0.75:0.3, by vol.) then (arrow) with chloroform:methanol:H_2O:trifluoroacetic acid (30:30:10:0.07, by vol.). (A) Radioactivity content of the eluates from the HPLC columns. (B) The PL1 and PL2 peaks obtained after HPLC were pooled and analyzed by gel electrophoresis as in fig.1.

complexes (PL1 and PL2) which are selectively phosphorylated by a calmodulin-dependent (PL1) or a cAMP-dependent (PL2) kinase. This observation rules out the possibility that the same protein chain contains 2 different sets of phosphorylatable sites. PL1 and PL2 display a number of common characteristics, like resistance to trypsin digestion and subunit composition. Their functional difference might be due to:

(i) A minor difference in their subunit composition (a minor low M_r accessory subunit?); or

(ii) A difference in the type and/or amount of tightly associated lipids.

The isolation and molecular analysis of PL1 and PL2 will be necessary to test these hypotheses. It will also be of great interest to investigate whether PL1 and PL2 are always present in equimolar amounts with the ATPase units to form a functional ternary complex, or whether two functionally distinct PL1–ATPase and PL2–ATPase complexes can be present into morphologically distinct regions of the SR network.

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