Early presence of phospholamban in developing chick heart

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Received 12 March 1983

Phosphorylation of phospholamban and development of reticular Ca^{2+} transport were studied in crude membrane preparations of embryonic, newborn and adult chick heart. Maximal phosphorylation of phospholamban by added catalytic subunit of cyclic AMP-dependent protein kinase increases from embryonic day 4–15. It decreases with further development. In the same membrane preparations active Ca^{2+}-uptake into vesicles of sarcoplasmic reticulum rises from day 4–7 and decreases then slightly until day 20. A several-fold increase in Ca^{2+}-transport activity occurs at the time of hatching. The data indicate separate genetic control for synthesis of phospholamban and sarcoplasmic reticulum Ca^{2+}-ATPase.

Cardiac muscle *Ontogenesis* *Sarcoplasmic reticulum* *Ca^{2+}-transport* *Phospholamban* *Protein kinase*

1. INTRODUCTION

Phospholamban, a protein of cardiac SR, is phosphorylated in perfused hearts in response to β-adrenergic catecholamines [1,2]. In isolated fragments of SR cyclic AMP-dependent phosphorylation of the protein is associated with increases in the velocity of active Ca^{2+}-transport and in the turnover of elementary steps of the Ca^{2+}-ATPase [3]. We now report that phospholamban is synthesized early during cardiac muscle ontogenesis. The protein is present in chick embryonic hearts when catecholamines are first capable of altering cardiac contractility. A parallel study of phospholamban phosphorylation and SR Ca^{2+}-uptake in crude membrane preparations revealed high tissue levels of the phosphoprotein and low activities of reticular Ca^{2+}-transport at developmental stages preceding hatching.

Abbreviations: SR, sarcoplasmic reticulum; cAMP-PrK, cyclic AMP-dependent protein kinase; C-subunit, catalytic subunit of cyclic AMP-dependent protein kinase; Ca-PrK, Ca^{2+}/calmodulin-dependent protein kinase; DTT, dithiothreitol; PMSF, phenylmethylsulfonylfluoride; M_r, relative molecular mass

2. MATERIALS AND METHODS

Hearts were removed from embryonated Leghorn chicken eggs, from newborn and from adult chickens. Crude membranes were prepared by disruption of whole hearts in 10 vol. 0.75 M KCl, 1 mM EDTA, 0.2 mM DTT, 0.1 mM PMSF, 5 mM histidine–HCl (pH 7.4), with a Polytron PT 10-35 at a setting of 6. Homogenization was performed 3-times for 10 s each. This was followed by centrifugation at 150000 × g for 1 h. The pellets obtained were rehomogenized and sedimented as before. After an additional wash with 0.2 mM DTT, 0.1 mM PMSF, 100 mM histidine–HCl (pH 7.4), membranes were suspended in 0.25 M sucrose, 10 mM histidine–HCl (pH 7.4). Fragmented SR was isolated as in [4].

Membrane phosphorylation with [γ-^{32}P]ATP (Amersham) was carried out for 1 min as in [5] in the absence or presence of either 0.5 μM C-subunit and 1 mM EGTA or 0.5 μM calmodulin and 0.1 mM $CaCl_2$. For inhibition of phosphoprotein phosphatases 15 mM NaF was included in the phosphorylation assay. In one series of experiments membranes were first treated with phosphoprotein phosphatase S in order to

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00145793/83/$3.00

dephosphorylate any phospholamban that might have been initially present in phosphorylated form. For this purpose, 1 mg membrane protein/ml was incubated for 30 min at 25°C in 160 mM KCl, 1 mM DTT, 50 mM Hepes–HCl (pH 7.4), with 0.3 mg phosphatase S partly purified through the ethanol and ammonium sulfate steps as in [6]. The amount of phosphatase S is sufficient to remove >90% of [^{32}P]phosphate from fully phosphorylated phospholamban. Treatment with phosphatase S was followed by 2 washes with KCl–Hepes buffer and suspension of membranes in 0.25 M sucrose, 10 mM histidine–HCl (pH 7.4). Phosphorylated membranes were solubilized in 5% SDS, 1% β-mercaptoethanol, 0.1 mM EDTA, 50 mM Tris–H_3PO_4 (pH 6.8) and heated for 1 min at 100°C. They were electrophorized in a SDS–urea–polyacrylamide system [7]. Following electrophoresis, gels were stained with Coomassie brilliant blue, destained, dried, and autoradiographed with ORWO HS 11 film. For quantitative evaluation respective areas were cut out from dried gels and counted by liquid spectrometry. M_r-markers were human albumin (67000), ovalbumin (43000), carbonic anhydrase (30000), soybean trypsin inhibitor (21500), cytochrome *c* (12800), aprotinin (6500) and glucagon (3480).

Oxalate-dependent Ca^{2+}-uptake was measured at 37°C in 40 mM imidazole–HCl buffer (pH 7.0) containing 100 mM KCl, 5 mM $MgCl_2$, 5 mM Na_2ATP, 10 mM K-oxalate, 10 mM NaN_3, 2 μM $NaVO_3$, 0.2 mM EGTA, 75 μM $^{45}CaCl_2$ (Amersham) of spec. act. 15 μCi/μmol and 40–80 μg membrane protein that had not been treated with phosphatase S. Reactions were started with ATP. They were stopped by millipore filtration (HAWP filters) followed by washing with 100 mM KCl, 1 mM $LaCl_3$, 40 mM imidazole–HCl (pH 7.0). Linear transport rates were calculated from data points at 0.5, 1, 2 and 3 min measured in triplicates. The uptake conditions allow for Ca^{2+}-accumulation into SR vesicles only. ATP-dependent Ca^{2+}-transport into sarcolemmal and mitochondrial vesicles is inhibited by $NaNO_3$ [8] and NaN_3 [9], respectively. To exclude possible effects of variation in membrane orientation Ca^{2+}-transport and protein phosphorylation were studied in the same membrane preparations. Both the active site of the Ca^{2+}-ATPase and the phosphorylation sites of phospholamban are exposed to the cytoplasmic side of SR membranes [3].

Homogeneous preparations of C-subunit [10] and calmodulin [11] were prepared by standard procedures. Protein inhibitor of cAMP-PrK was a generous gift of Dr H. Haase from our laboratory. The inhibitor was purified 1200-fold from rabbit skeletal muscle [12]. Protein was determined as in [13] with ovalbumin as standard.

3. RESULTS AND DISCUSSION

Fig.1A demonstrates the electrophoretic behavior of phosphoproteins contained in crude membranes collected quantitatively from chick heart homogenates as compared to phosphoproteins in purified chick heart SR. A major phosphoprotein in both membrane preparations is phospholamban. The protein was identified by two criteria:

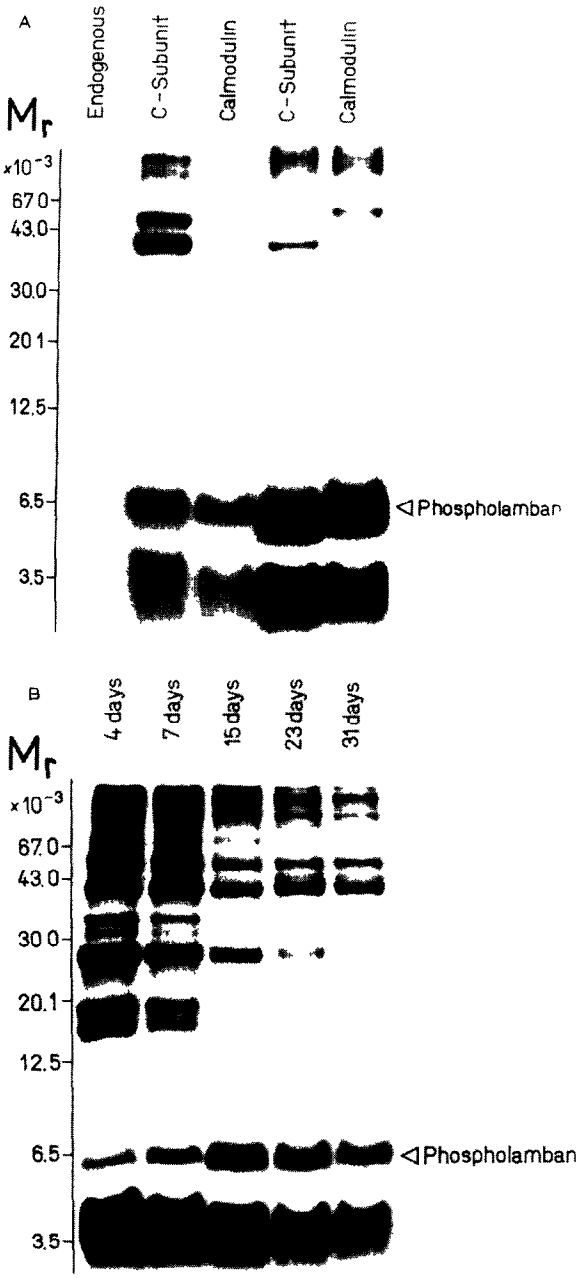
(i) A characteristic change in electrophoretic mobility occurring during heating in the presence of SDS [15].
(ii) The effective phosphorylation by cAMP-PrK and Ca-PrK [16].

Heat treatment disaggregates phospholamban oligomers of M_r 20000–24000 into subunits of M_r 5500–9000. The M_r of the dissociated phosphopolypeptide as estimated in the electrophoretic system in [7] is 6000 (fig.1). Phosphorylation of the polypeptide is catalyzed by added C-subunit and by a membrane-bound Ca-PrK. It was also found that the two phosphorylations were additive.

The extension of phosphoprotein analysis to membrane mixtures of developing hearts revealed that phospholamban is present in cardiac membranes of all developmental stages from embryonic day 4 onwards (fig.1B,C). For the early embryonic stages this conclusion relies mainly on experiments with C-subunit. The high amount of enzyme added assures an almost complete phosphorylation of available phospholamban molecules within the reaction time. [^{32}P]Phosphate incorporated into phospholamban/mg membrane protein increases from embryonic day 4–15. It decreases with further development (table 1). When phospholamban phosphorylation by C-subunit is related to a unit of heart mass, the largest changes take place again

in the early embryonic period. Calculated values for nmol [^{32}P]phospholamban/g wet heart wt increase from 84.6 at day 4 to 480.3 at day 15. Further development is associated with only moderate increases and a drop in phospholamban phosphorylation in aged myocardium. Phosphorylation with C-subunit was also carried out after prior dephosphorylation of membranes with phosphoprotein phosphatase S. [^{32}P]Phosphate incorporation into the M_r 6000 polypeptide changes only little following such treatment (table 1). The data indicate that phospholamban is present in isolated membranes largely in dephosphorylated form. Maximal phosphorylation with C-subunit may therefore be used as a method for rough estimation of the amount of phospholamban exposed to the medium. The method does not allow for determination of phospholamban inside closed membrane vesicles.

Upon addition of Ca^{2+} and calmodulin an endogenous Ca-PrK becomes activated. The activity of the enzyme varies with development. Ca^{2+}/calmodulin-dependent phosphorylation of phospholamban as compared to phosphorylation by C-subunit is very low in membranes of early embryonic hearts (fig.1, table 1). Moreover, in membranes of 4-day-old and also of 6-day-old em-



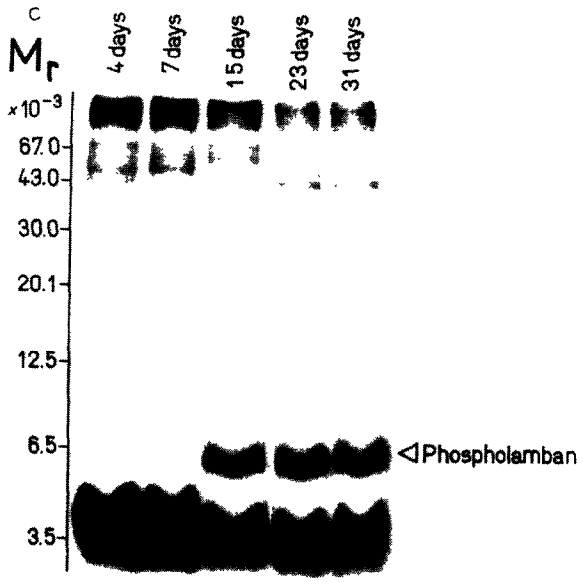


Fig.1. SDS–polyacrylamide gel electrophoresis and autoradiography of phosphorylated chick heart membranes: (A) phosphoproteins in a crude membrane preparation (a–c) and in SR (d,e) prepared from adult heart; (B) phosphorylation of crude membranes from developing hearts by C-subunit; (C) Ca^{2+}/calmodulin-dependent phosphorylation of crude membranes from developing hearts. Embryonic ages were determined by comparison with the description in [14]. Hatching occurs on day 21. The amount of protein applied/lane was 100 μg for crude membranes and 30 μg for purified SR.

Table 1

Phospholamban phosphorylation and oxalate-supported Ca^{2+}-uptake in mixed membrane preparations of embryonic, developing and adult chick heart

Developmental stage (days):	Embryonic period 4	7	15	20	Postnatal period 23	31	>500
Membrane protein (mg/g wet heart wt)	6.0 ± 0.8	3.8 ± 0.5	8.8 ± 1.0	14.5 ± 1.2	18.6 ± 1.0	20.2 ± 1.0	18.9 ± 1.4
[^{32}P]Phospholamban (pmol ^{32}P/mg membrane protein)							
Phosphorylation in the presence of C-subunit	14.1 ± 1.0	27.2 ± 2.3	54.8 ± 2.9	34.0 ± 3.2	38.3 ± 1.5	28.3 ± 2.2	18.4 ± 2.5
Phosphorylation in the presence of Ca^{2+} and calmodulin	1.4 ± 0.3	4.5 ± 0.7	28.8 ± 4.7	38.0 ± 3.2	28.9 ± 2.7	21.3 ± 4.1	10.8 ± 2.2
Phosphorylation of phosphatase S-treated membranes with C-subunit	n.d.	n.d.	49.7 ± 2.2	35.9 ± 2.0	40.0 ± 5.1	38.4 ± 2.9	18.7 ± 3.2
Oxalate-supported Ca^{2+}-uptake (nmol Ca^{2+}. min^{-1}. mg membrane $protein^{-1}$)	1.7 ± 0.2	9.2 ± 2.0	8.2 ± 2.3	6.1 ± 1.6	23.0 ± 1.7	24.0 ± 6.2	12.6 ± 2.1

Means ± SE were calculated from experimental data obtained with 3 different membrane preparations; n.d., not determined. For further experimental details see section 2

bryos, phospholamban phosphorylation measured in the presence of Ca^{2+} and calmodulin is reduced further by about 60% with 2.8 μg/ml of partly purified protein inhibitor of cAMP-PrK (not shown). The inhibited activity should be attributed to endogenous cAMP-PrK. It is evident then, that rather low activities of Ca-PrK are present before day 7. Only at later developmental stages phosphorylation of phospholamban is effectively catalyzed by a membrane-bound Ca^{2+}-dependent enzyme. On the contrary, cAMP-PrK is present in high activities in early embryonic hearts. The activity of this kinase decreases with development [17]. Retardation in the synthesis of Ca-PrK during ontogenesis thus offers a unique opportunity for studies aimed at the physiological relevance of cyclic AMP-dependent as opposed to Ca^{2+}/calmodulin-dependent phosphorylation of phospholamban.

In parallel with phosphorylation, Ca^{2+}-transport activities of SR fragments were evaluated in mixed membrane preparations (table 2). Ca^{2+}-transport activities were determined from linear rates of ATP-dependent Ca^{2+}-uptake measured in the presence of oxalate. The rate values are proportional to Ca^{2+}-ATPase activity [18]. Oxalate-supported Ca^{2+}-uptake is low in membranes from 4-day old embryos. It rises from day 4–7 and then decreases slightly until day 20. At the time of hatching another steep increase occurs. The large enhancement in Ca^{2+}-transport activity between embryonic day 20 and day 2 of postnatal life is accompanied by only minor changes in cardiac phospholamban.

The early presence of phospholamban in embryonic hearts is in line with the hypothesis that the protein is involved in β-adrenergic regulation of cardiac contraction. Catecholamines are first capable of altering contractility in chick embryonic hearts at day 4 [19]. The effects are associated with a rise in cellular cyclic AMP [20]. Changes in tension development elicited by catecholamines

before day 5 are, however, rather small: between 30–50%. With increasing age β-adrenergic effects become manifest: a 3–4-fold increase in peak-twitch tension and maximal rise of tension development are characteristic actions of saturating doses of isoproterenol in ventricles of 12-day-old embryos [19]. At the same developmental stages there occurs a large increase in cardiac phospholamban (fig.1, table 1). These changes take place before the onset of adrenergic neuroeffector transmission. Adrenergic innervation is not operative in embryonic chick heart before day 16 [21].

Experiments with isolated SR suggest that phospholamban is involved in the regulation of reticular Ca^{2+}-ATPase. A close functional and spatial relationship of both proteins has been proposed (reviewed in [3]). The present results demonstrate a dissociation of phospholamban synthesis and development of reticular Ca^{2+}-transport during ontogenesis. The observation does not rule out possible regulations of SR Ca^{2+}-ATPase by phospholamban. It demonstrates, however, that synthesis of the two proteins is under separate genetic control.

ACKNOWLEDGEMENT

We thank John Huggins for critical reading of the manuscript.

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