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ISOLATION OF PHOSPHOLAMBAN AND A SECOND PROTEOLIPID COMPONENT FROM CANINE CARDIAC SARCOPLASMIC RETICULUM

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Summary:

We have isolated two proteolipid fractions from canine cardiac sarcoplasmic reticulum by chromatography on columns of Sepharose CL-6B, and Sephadex LH-60. One, "fraction B", is phosphorylated by cyclic AMP-dependent protein kinase and was identified as phospholamban, the activator of cardiac sarcoplasmic reticulum. The other, "fraction A", is not phosphorylated and has an amino acid composition very similar to those of proteolipids we previously isolated from (Na,K)-ATPase.

Introduction:

The Ca^{2+}-ATPase of cardiac SR[1] is activated, and Ca^{2+} transport is stimulated, upon phosphorylation of a small, membrane-bound protein that has been named phospholamban (1). It has been known for several years that phospholamban can be phosphorylated by either endogenous or exogenous cAMP-dependent protein kinases (2-5). Recently, it has been reported (6-8) that phospholamban is also phosphorylated, at different sites on the protein, by an endogenous, membrane-bound, Ca^{2+}-dependent, calmodulin-stimulated protein kinase. According to SDS-polyacrylamide gel electrophoresis, phospholamban has $M_r \sim 22,000$. One group, however, has recently presented evidence that this form is a dimer (6, 9) of $M_r \sim 11,000$ subunits. These authors further reported that phospholamban is a proteolipid. Proteolipids are very

[1] Abbreviations: SR, sarcoplasmic reticulum, cAMP, cyclic adenosine 3',5'-monophosphate; SDS, sodium dodecylsulfate.

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hydrophobic proteins which are characterized by their solubility in chloroform: methanol mixtures (10); in some, but not all, cases they contain covalently attached lipids (11). The finding that phospholamban is a proteolipid suggested to us that it might be related to the $M_r \sim 12{,}000$ proteolipids that we had recently isolated from lamb kidney (Na,K)-ATPase by column chromatographic procedures (12). We describe here the application of these procedures for the isolation of two proteolipid components from canine cardiac SR, one of which we have identified as phospholamban. While this work was being completed, Le Peuch, et al. (13) published an amino acid composition for phospholamban which differs from that reported here.

Experimental Procedures:

SR from dog cardiac muscle was prepared by a modification of the Harigaya and Schwartz procedure (14) as previously described (15). The final yield of SR protein ranged from 0.6 to 0.8 mg per gram of wet cardiac tissue. The purity and homogeneity of the preparations was checked by SDS-polyacrylamide gel electrophoresis and by various enzyme marker activities. Sarcolemmal and mitochondrial contamination were low, as judged from 5'-nucleotidase (0.043 ± 0.015 μmol mg^{-1} min^{-1}), H^3-ouabain binding (4-10 pmoles/mg), and cytochrome c oxidase (0.0058 μmol mg^{-1} min^{-1}) activities. The SR Ca^{2+}-ATPase (50 μg of protein/ml) was assayed in 25 mM histidine buffer (pH 7.0) containing 5 mM $MgCl_2$, 100 mM KCl, 5 mM NaN_3, 100 μM EGTA and 100 μM $CaCl_2$ (10 μM free Ca^{2+}) and 5 mM ATP; the specific activity was 0.67-1.00 μmol P_i mg^{-1} min^{-1} at 37°C. The rate of Ca^{2+} uptake was determined at 20°C in 40 mM Tris/maleate buffer (pH 6.8) containing 100 mM KCl, 10 mM $MgCl_2$, 5 mM NaN_3, 5 mM Tris-oxalate, 5 mM ATP, 100 μM EGTA, and 100 μM $CaCl_2$ (10 μM free Ca^{2+}) and found to be 0.33-0.42 μmol mg^{-1} min^{-1}.

Phosphorylation of SR was carried out at 30°C for 5 min in 50 mM phosphate buffer (pH 7.0) containing 10 mM $MgCl_2$, 10 mM NaF, 1 mg/ml SR vesicles and 500 μM ATP (including [γ-^{32}P]ATP), in the presence of cAMP (1 μM) and cAMP-dependent protein kinase (0.1 mg/ml). The reaction mixture was then centrifuged at 105,000 x g for 30 minutes. The pellet was homogenized gently in ice-cold 20 mM Tris/maleate buffer (pH 6.8) containing 100 mM KCl to a concentration of 7-12 mg protein/ml. Then solid SDS (10 mg per mg of protein) and β-mercaptoethanol (final concentration 1%) were added, and the mixture was stirred for 1 hour at 37°C. The solubilized SR was then dialyzed overnight at room temperature vs column buffer (see Fig. 1) and 40-100 mg of protein, at a concentration of 2-4 mg per ml, was applied to a 5 x 195 cm column of Sepharose CL-6B. Fractions from this column were lyophilized, and the ^{32}P-containing fraction was dissolved in 3-4 ml of 88% formic acid and applied to a 2 x 195 cm column of Sephadex LH-60. The LH-60 column was run in a 3:1 (v/v) mixture of 95% ethanol and 88% formic acid, a solvent very similar to that developed by Gerber, et al. (16) for separation of hydrophobic peptides.

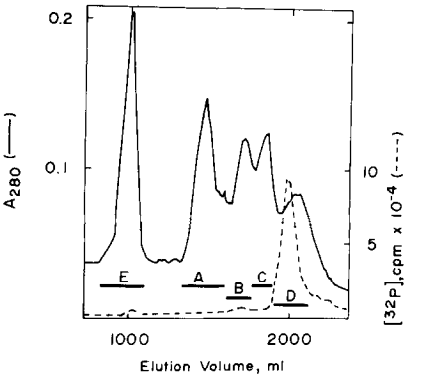

Figure 1: Separation of the components of solubilized canine cardiac SR on a 5 x 195 cm column of Sepharose CL-6B. Solvent: 5 mM sodium phosphate, pH 8.0, containing 0.1% SDS, 1 mM 2-mercaptoethanol and 0.01% sodium azide. Fractions of 10 ml each were collected at a flow rate of 80 ml/hr. Horizontal bars indicate fractions pooled.

Amino acid analyses were carried out on a Glenco MM-60 amino acid analyzer by standard procedures. Samples for analysis were evacuated and hydrolyzed at 110°C for 20 hr in 6N HCL containing 0.1% phenol. Phospholipids were detected by the presence of large amounts of serine and ethanolamine in hydrolyzed samples. (On our amino acid analyzer, ethanolamine is eluted between lysine and ammonia. Accurate quantitation of lysine was not possible in the presence of a large excess of ethanolamine). Protein concentrations were determined by amino acid analysis. SDS-polyacrylamide gel electrophoresis was carried out according to the method of Laemmli (17).

Results and Discussion:

The results of gel filtration of solubilized cardiac SR on Sepharose CL-6B are shown in Fig. 1. The separation pattern is very reproducible and has been obtained with seven different preparations. Each of the fractions indicated was examined by electrophoresis on 0.1% SDS-7.5% polyacrylamide gel electrophoresis and by amino acid analysis. Fraction A, which we presume to be the Ca^{2+}-ATPase protein (18), accounted for 33% of the total protein recovered and contained a single major component (at least 90% pure) of $M_r \sim 100,000$. Fraction E, the large peak at the void volume of the column, accounted for only 5% of the total protein recovered and contained no components that stained with Coomassie blue after electrophoresis. Fraction D contained essentially all of the phosphoprotein.

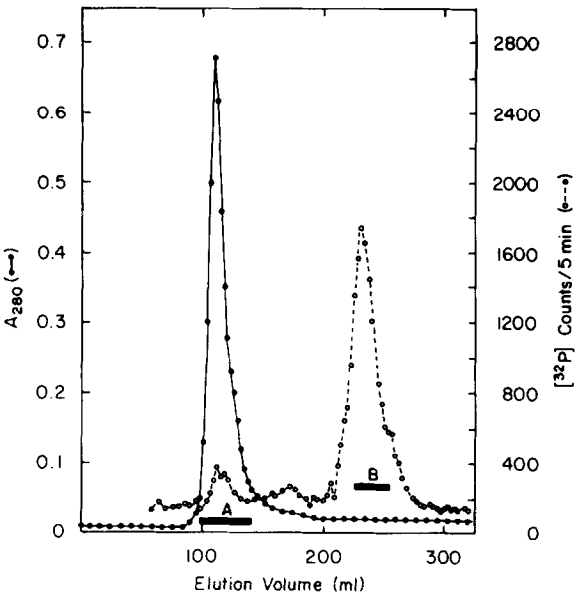

Figure 2: Chromatography of fraction D from Figure 1 on a 2 x 95 cm column of Sephadex LH-60. Solvent: 88% formic acid:95% ethanol (1:3, v/v). Fractions of 3 ml each were collected at a flow rate of 20 ml/hr. Horizontal bars indicate fractions pooled.

The results of Sephadex LH-60 chromatography of the phosphoprotein-containing material is shown in Figure 2. Two major proteolipid fractions, designated A and B, were obtained. Fraction B was identified as phospholamban by the appearance of a single band of $M_r \sim 22,000$ on autoradiograms of 0.1% SDS-12.5% polyacrylamide gel electrophoretograms. This is the same band that we observe (7) after electrophoresis of ^{32}P-labeled SR. When the gels were stained with Coomassie blue, fraction B again showed a single major band of $M_r \sim 22,000$, while fraction A revealed a very weakly staining band of $M_r \sim 11,000$. Extremely faint bands of $M_r \sim 15,000$ and $M_r \sim 20,000$ could also be observed on stained gels of fraction A, indicating that this fraction is not homogeneous. In earlier experiments (not shown) we extracted the phosphoprotein-containing fraction of Figure 1 with the chloroform-methanol solvent that we used previously to isolate proteolipids from lamb kidney Na,K-ATPase (12). When the chloroform-methanol extract from cardiac

TABLE I
Amino Acid Compositions of Proteolipids from Cardiac Sarcoplasmic Reticulum.[a]

	Fraction A	Fraction B	"Phospholamban"[b]
Asp	8.24	8.93	11.1
Thr	5.66	4.79	5.5
Ser	7.05	5.75	5.5
Glu	11.2	16.7	11.4
Pro	5.88	2.34	5.5
Gly	7.69	1.85	10.6
Ala	8.22	8.55	11.0
Val	5.69	5.08	6.5
Met	2.33	5.90	1.7
Ile	4.68	9.77	4.9
Leu	10.6	13.3	9.5
Tyr	3.57	2.29	2.1
Phe	5.42	3.68	3.4
Lys	6.65	2.51	4.9
His	2.30	<0.1	2.0
Arg	4.89	8.63	4.4
Yield[c]	46	10	5

[a]Expressed as residues per 100 residues. Fractions A and B are the peaks shown in Figure 2. Cys and Trp were not determined.
[b]From Le Peuch, et al.; Cys not present, Trp not determined (13).
[c]Expressed as μg recovered per mg of SR protein.

SR was chromatographed on Sephadex LH-60 using the same solvent, we also obtained the fractions A and B shown in Figure 2, although the yields of fraction A were about 50% lower.

The recoveries and amino acid compositions of fractions A and B of Figure 2 are listed in Table I. It is noteworthy that fraction B, which contains most of the phosphoprotein, represents less than 20% of the total protein recovered. The low content of histidine in fraction B is a further indication of homogeneity. When only the middle third of the fraction B peak was pooled, and then rechromatographed on the same column, its amino acid composition did not change significantly. Fraction A has a distinctly different composition from fraction B, particularly in the content of glutamic acid, glycine, methionine, isoleucine, and the basic amino acids lysine, histidine and arginine.

Most groups report that phospholamban is a protein of $M_r \sim 22{,}000$. Recently, however, Le Peuch, et al. (13) reported the purification of canine

TABLE II

Statistical Comparisons of Proteolipids Associated with Sarcoplasmic Reticulum and (Na,K)-ATPase.[a]

	2.[b]	3.[c]	4.[d]	5.[e]	6.[f]
1. fraction A	167	39	38	31	26
2. fraction B	0	211	111	189	210
3. "Phospholamban"		0	47	57	45
4. Cardiac (Na,K)-ATPase.			0	77	61
5. Kidney (Na,K)-ATPase $\gamma 1$				0	56
6. Kidney (Na,K)-ATPase $\gamma 1$					0

[a]Individual differences in moles% for each amino acid (excluding Cys and Trp) were squared and summed to obtain the tabulated values (20). Lower numbers are indicative of greater similarity in amino acid composition, with values below 50 indicating a high probability of amino acid sequence homology. [b]Fractions A and B of cardiac SR are the peaks shown in Figure 2. [c]From canine cardiac SR (13). [d]The 11,700-dalton phosphorylated protein isolated from beef heart (Na,K)-ATPase by Dowd, et al. (19). [e]The two proteolipid fractions from lamb kidney (Na,K)-ATPase (12).

cardiac phospholamban by a two-dimensional gel electrophoresis technique. In the first dimension, a radioactive band of $M_r \sim 22,000$ (which they call the dimeric form of phospholamban) was isolated in the presence of SDS. For the second dimension, the presumed monomeric, $M_r \sim 11,000$ form was isolated after dissociation in Triton X-100 and SDS. We have not been able to reproduce this apparent dissociation of phospholamban on gel electrophoresis. Surprisingly, the amino acid composition of the "phospholamban" preparation of Le Peuch, et al. (see Table I) is similar to our fraction A and very different from our phosphorylated fraction B. The reason for this discrepancy is unknown. It is possible that their preparation consists mainly of material equivalent to our fraction A, plus a small amount of fraction B, and perhaps other material which we have removed by chromatography on Sephadex LH-60. They were not able to stain their preparation with Coomassie blue after gel electrophoresis; this is consistent with our finding that fraction A stains very weakly compared to fraction B.

In Table II the amino acid compositions of fractions A and B are compared with the "phospholamban" of Le Peuch et al. (13), two proteolipids (γ_1 and γ_2)

of $M_r \sim 12,000$ that we recently isolated from lamb kidney (Na,K)-ATPase (12), and a $M_r \sim 11,700$ phosphorylated protein of unknown function isolated from bovine cardiac (Na,K)-ATPase (19). While all of the proteolipids except fraction B appear to be very similar, any speculation regarding possible amino acid sequence homology would be premature since further work is needed to unambiguously establish their purity. Nevertheless, it will be very intersting to compare the amino acid sequences of these and other proteolipids associated with membrane ATPases.

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References:

1. Tada, M., Kirchberger, M.A., Iorio, J.M., and Katz, A.M. (1973) Circulation 48: Suppl. 4, 25.
2. LaRaia, P.J., and Morkin, E. (1974) Circ. Res. 35: 298-306.
3. Wray, H.L., and Gray, R.R. (1977) Biochim. Biophys. Acta 461: 441-459.
4. Schwartz, A., Entman, M.L., Kaniike, K., Lane, L.K., Van Winkle, W.B., and Bornet, E.P. (1976) Biochim. Biophys. Acta 426: 57-72.
5. Kirchberger, M.A., and Tada, M. (1976) J. Biol. Chem. 251: 725-729.
6. Le Peuch, C.J., Haiech, J., and DeMaille, J.G. (1979) Biochem. 18: 5150-5171.
7. Kranias, E.G., Bilezikjian, L.M., Potter, J.D., Piascik, M.T. and Schwartz, A. (1980) Ann. N.Y. Acad. Sci.: in press.
8. Bilezikjian, L.M., Kranias, E.G., Potter, J.D. and Schwartz, A. (1980) Fed. Proc. 39: 1663.
9. Le Peuch, C.J., Le Peuch, D.A.M., and DeMaille, J.G. (1980) Fed. Proc. 39: 1817.
10. Folch, J., and Lees, M. (1951) J. Biol. Chem. 191: 807-817.
11. Folch-Pi, J., and Sakura, J.D. (1976) Biochim. Biophys. Acta 427: 410-427.
12. Reeves, A.S., Collins, J.H., and Schwartz, A. (1980) Biochem. Biophys. Res. Commun., 95: 1591-1598.
13. Le Peuch, C.J., Le Peuch, D.A.M., and DeMaille, J.G. (1980) Biochemistry 19: 3368-3373.

14. Harigaya, S., and Schwartz, A. (1969) Circ. Res. 25: 781-794.
15. Sumida, M., Wang, T., Mandel, F., Froehlich, J.P., and Schwartz, A. (1978) J. Biol. Chem. 253: 8772-8777.
16. Gerber, G.E., Anderegg, R.J., Herlihy, W.C., Gray, C.P., Biemann, H. and Khorana, H.G. (1979) Proc. Natl. Acad. Sci., U.S.A. 70: 227-231.
17. Laemmli, U.K. (1970) Nature 227: 680-685.
18. Van Winkle, W.B., Pitts, B.J.R., and Entman, M.L. (1978) J. Biol. Chem. 253: 8671-8673.
19. Dowd, F.J., Pitts, B.J.R., and Schwartz, A. (1976) Arch. Biochem. Biophys. 175: 321-331.
20. Weltman, J.H., and Dowben, R.M. (1973) Proc. Natl. Acad. Sci. U.S.A. 70: 3230-3234.