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ISOLATION OF CARDIAC MEMBRANE PROTEOLIPIDS BY HIGH PRESSURE LIQUID CHROMATOGRAPHY

A COMPARISON OF RETICULAR AND SARCOLEMMAL PROTEOLIPIDS, PHOSPHOLAMBAN AND CALCIDUCTIN

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Membrane-bound phosphorylatable proteolipids were reported to play a role in the regulation of transmembrane Ca^{2+} fluxes by catecholamines. A generally applicable purification procedure is described by which such proteolipids as the cardiac sarcoplasmic reticulum phospholamban is purified by solvent extraction followed by high pressure liquid chromatography on microparticulate silica. Phospholamban is thereby purified with a yield of 3.37 mg from 100 mg of sarcoplasmic reticulum proteins, significantly higher than that obtained by any of the previously reported procedures. It appeared homogeneous upon dodecyl sulfate-polyacrylamide gel electrophoresis where it is stained by Coomassie blue and detected by autoradiography. The same procedure is applicable to cardiac sarcolemmal calciductin. Both proteolipids exhibit the same M_r 11 000 and p*I* 3.7 upon two-dimensional gel electrophoresis. Their amino acid compositions are very similar if not identical. This raises the intriguing possibility that phospholamban and calciductin are identical though they obviously belong to different membranes.

Introduction

Epinephrine and other β-adrenergic agonists are known to increase the cardiac contractile force (positive inotropic effect) and to abbreviate systol as part of the positive chronotropic effect (acceleration of the cardiac rythm). Such β-agonists act through binding to specific membrane receptors and activating the membrane-bound adenylate cyclase, resulting in cAMP synthesis and activation of cAMP-dependent protein-kinases [1,2]. The latter are capable of modulating Ca^{2+} fluxes through sarcolemmal and reticular membranes by catalyzing the phosphorylation of water-insoluble membrane proteins, the proteolipids. The first well documented example was the activation of the cardiac sarcoplasmic reticulum calcium pump brought about by cAMP-dependent phosphorylation of phospholamban [3,4], which was later shown to be also a substrate of a membrane-bound, calcium/calmodulin-dependent phospholamban kinase [5,6]. Such phosphorylations increase the rate of Ca^{2+} uptake by the cardiac sarcoplasmic

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Abbreviations: SDS, sodium dodecyl sulfate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; cAMP, cyclic adenosine 3′:5′-monophosphate; C subunit, the catalytic subunit of cAMP-dependent protein-kinase; Nonidet P40, ethylphenylpolyethyleneglycol, NP40; acidic chloroform/methanol, chloroform/methanol/4 M HCl (200:100:0.75, v/v).

reticulum and account for the systole abbreviation. More recently, other examples of membrane phosphorylatable proteolipids have been described. Calciductin is the major phosphate-acceptor of cardiac sarcolemma [7] and its cAMP-dependent phosphorylation is linearly correlated with the cAMP-dependent increase in the rate of ATP-independent Ca^{2+} influx into sarcolemmal vesicles [8]. Comparison between cAMP-dependent protein kinase substrates in sarcoplasmic and sarcolemmal vesicles have already been carried out [9–11] and phospholamban or phospholamban like proteolipids described in both vesicles. Similarly, platelet calcium-accumulating vesicles contain a phospholamban-like protein that is phosphorylatable by cAMP-dependent protein kinase [12], with alteration of Ca^{2+} fluxes.

Membrane phosphorylatable proteolipids exhibit a similar molecular weight of approx. 23000. Phospholamban was shown to dissociate into lower M_r subunits of 11000 [13] and even 5500 [14]. Similarly calciductin is present in two interconvertible forms of M_r 23000 and 11500 [8,15]. It is therefore tempting to speculate that the above proteolipids, though belonging to different membranes, are related or perhaps identical in structure, a hypothesis which will be checked only after their purification to homogeneity.

A first step toward the isolation of phospholamban was the description of a diagonal electrophoretic procedure that, though tedious, was useful in allowing the physicochemical characterization of the proteolipid [13]. This report describes a more generally applicable approach to the purification of phosphorylatable membrane proteolipids which takes advantage of their solubility in organic solvent mixtures. The solvent extract is then submitted to high performance liquid chromatography on microparticulate silica. Such techniques allowed a better comparison of phospholamban and calciductin, which appeared to comigrate in two-dimensional gel electrophoresis and to exhibit a similar amino acid composition.

Materials and Methods

Miscellaneous: Carrier-free [^{32}P]orthophosphoric acid was from New England Nuclear. [γ-^{32}P]ATP (spec. act. 400 to 600 cpm/pmol) was prepared according to Glynn and Chappell [16]. The catalytic subunit (C subunit) of cAMP-dependent protein kinase (type II from bovine heart) was prepared and stored as described previously [17]. Protein concentrations were determined by the Coomassie blue technique [18]. Ampholytes were from Serva. Nonidet P40 was obtained from Fluka, and ultrapure grade urea from Schwarz/Mann. Polyacrylamide gel electrophoresis reagents were from BioRad and Serva, and the electrophoresis calibration kit was obtained from Pharmacia. Stock solutions of acrylamide were filtered through 0.22 μm Millipore filters. Chemicals and solvents were reagent grade and usually obtained from Merck.

Preparation of canine cardiac sarcolemmal vesicles. Sarcolemmal vesicles were isolated from diastole-arrested canine heart essentially as described in Ref. 19, and suspended in 20 mM Hepes buffer (pH 7.3) at a final protein concentration of 7 mg/ml. The yield was approx. 10 mg of proteins per 100 g of starting material and the preparation was stored frozen at -20°C when not used immediately. The membrane preparation was characterized by measuring the $(Na^+ + K^+)$-ATPase and 5′-nucleotidase activities, the initial rate of the ATP-dependent Ca^{2+}-uptake and the maximal calcium accumulation into the vesicles in the presence of 5 mM oxalate, as in Ref. 7.

Preparation of canine cardiac sarcoplasmic reticulum vesicles. Sarcoplasmic reticulum vesicles were prepared from dog heart ventricles as described previously [5], and stored frozen at -20°C. The final yield was 1 mg of sarcoplasmic reticulum protein per g of ventricular tissues.

Phosphorylation of sarcolemmal and sarcoplasmic reticulum vesicles. cAMP-dependent phosphorylations of sarcolemma and sarcoplasmic reticulum vesicles were carried out as in Ref. 8 at 20°C, in 0.05 mM dithiothreitol, 10 mM NaF, 50 mM sodium phosphate buffer (pH 7.0) containing 1 to 4 mg of sarcolemma or 4 to 20 mg of sarcoplasmic reticulum protein per ml. 10 min after addition of catalytic of cAMP-dependent protein kinase ($E/S = 0.01$), 10 mM magnesium acetate and 0.3 mM [γ-^{32}P]ATP (400–600 cpm/pmol), the phosphorylation reaction was quenched by addition of either 1% (w/v) sodium dodecyl sulfate (SDS) for poly-

acrylamide gel electrophoresis samples, or 20 mM EDTA before proteolipid extraction.

Proteolipid extraction. Phosphorylated sarcoplasmic reticulum (80 mg) or sarcolemma (10 mg) vesicles were centrifuged at 100 000 × *g* for 60 min. The pellet was suspended in 5 mM EDTA, 20 mM NaF, 50 mM sodium phosphate buffer, pH 7.0, and centrifuged again 100 000 × *g* for 1 h. The pellet was resuspended in 0.5% (w/v) ammonium bicarbonate and centrifuged at 100 000 × *g* for 1 h, then suspended in a minimum volume of 0.5% (w/v) ammonium bicarbonate and lyophilized. Proteolipids were extracted from lyophilized vesicles by chloroform/methanol/4 M HCl (200 : 100 : 0.75, v/v) essentially as described in Ref. 13. 5 ml of solvent were added and the mixture kept under stirring for 10 min at room temperature. The suspension was centrifuged 10 min in a clinical centrifuge and the extraction repeated four times. Extracts were combined and stored at −20°C until use.

High pressure liquid chromatography. High pressure liquid chromatography purifications were performed with a Waters high performance liquid chromatograph equipped with a pump model 6000 A and an universal injector model U6K. Solvents were pumped onto a silica column (4.6 mm × 15 cm) packed with Spherisorb S5W obtained from Chrompack. All separations were performed at room temperature, at a flow-rate of 1 ml/min. Aliquots (1 ml) of the proteolipid extract were injected onto the silica column, equilibrated in chloroform/methanol/water/trifluoroacetic acid (200 : 100 : 0.75 : 0.3, v/v). The column was washed with 40 ml of this solvent, then with chloroform/methanol/water/trifluoroacetic acid (30 : 30 : 10 : 0.07, v/v). Fractions were collected and counted in a liquid scintillation counter by using the Cerenkov radiation. Radioactive fractions were dried under nitrogen and processed for gel electrophoresis or amino acid analysis.

Mono-dimensional polyacrylamide gel electrophoresis. 0.1% SDS-15% polyacrylamide slab gel electrophoresis was carried out according to Laemmli [20], using analytical 1.5 mm thick gels. Electrophoretograms were stained with Coomassie blue, destained, dried, and autoradiographed with Kodak Mini-R films.

Two-dimensional polyacrylamide gel electrophoresis. [^{32}P]Phosphate-labeled proteolipids were extracted from 4 mg of sarcolemma or sarcoplasmic reticulum vesicles, three times with 0.5 ml of acidic chloroform/methanol (2 : 1, v/v) as described above. The combined extracts were dried under nitrogen and treated 45 min at room temperature in 100 μl of the lysis buffer of O'Farrell [21]. The resulting suspensions were clarified by centrifugation at 20 000 × *g* in a Beckman microfuge. Two-dimensional gel electrophoresis was performed as described by O'Farrell [21] on supernatant aliquots (50 μl). Isoelectric focusings, using 1.6% (v/v) ampholytes pH range 2–4 and 0.4% (v/v) ampholytes pH range 2–11, were carried out on 1.85 mm × 9 cm gels which were run according to the following schedule: 200 V for 15 min, 300 V for 30 min, 400 V for 13 h and 800 V for 1 h. Samples were run in duplicate, and one of the gels was cut into 1 cm long pieces soaked 30 min in 0.5 ml H_2O for pH measurements. Isoelectric focusing gels were equilibrated 2 h in 2 ml of sample buffer: 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, 2.3% (w/v) SDS, 0.1% (w/v) Bromophenol blue, 0.0625 M Tris-HCl (pH 6.8), then layered at the top of 0.1% SDS-15% polyacrylamide slab gels without stacking according to Laemmli [20]. Gels were run at a constant voltage of 170 V, and processed as described above for mono-dimensional gels.

Amino acid and phosphoamino acid analysis. Amino acid analysis were carried out on a Beckman Multichrom amino acid analyzer according to Moore and Stein [22], after 24 h hydrolysis at 110°C with constant boiling HCl in evacuated and sealed tubes. Amino acids were detected after post-column reaction with ninhydrin.

[^{32}P]Phosphate-labeled *o*-phosphoamino acids were identified after partial acid hydrolysis of the labeled proteins in constant boiling HCl at 110°C, for various times [23]. Separation of [^{32}P]phosphate-labeled *o*-phosphoamino acids with or without addition of unlabeled standards was carried out on the short column of the amino acid analyzer, by using 10 mM trifluoroacetic acid as described elsewhere [8,24]. Phosphoamino acids were detected after post-column derivatization with *o*-phthaldialdehyde, and the corresponding fractions, collected every minute, were counted by using the Cerenkov radiation.

Results

The diagonal electrophoretic procedures previously used in this laboratory for the purification of phospholamban [13], was rather tedious and gave only limited amounts of the proteolipid. Phospholamban was obtained with a 6% yield after extraction from sarcoplasmic reticulum vesicles, electrophoresis on a 0.1% SDS-15% polyacrylamide gel, dissociation of the M_r 22000 form into a M_r 11000 form by treatment with the non-ionic detergent Triton X-100, electrophoresis on a second gel, and finally electrophoretic elution from the last gel. Major losses occurred through incomplete extraction of sarcoplasmic reticulum vesicles, incomplete dissociation of the phospholamban-ATPase complex, and poor recovery from the gel by electrophoretic elution. Phospholamban isolated by this method was unstained by Coomassie blue and showed one main radioactive band of M_r 11000 together with a faint radioactive band of M_r approx. 6000. Its amino acid composition was compatible with a minimum molecular weight of 5500 and the amount of protein recovered was 400 μg starting from 80 mg of sarcoplasmic reticulum protein. It was however clear from these studies that phosphorylated phospholamban was efficiently extracted into organic solvents and that purification procedures taking into account this property had to be developed. Chloroform/methanol/4 M HCl (200 : 100 : 0.75, v/v) was chosen since 75% of phospholamban is extracted by this solvent mixture [13]. In preliminary experiments, precipitation of proteolipids by addition of 5 volumes of diethyl ether, as described in Refs. 25 and 26, to remove lipids, was used, but redissolution of the proteolipid was difficult, and lower final yields were obtained. Ion exchange chromatography in organic solvents according to Ref. 27, and adapted as in Ref. 28 for proteolipid purification was unsuccessful. Gel filtration on LH 60 either in acidic chloroform/methanol or in mixtures of formic acid and ethanol as in Ref. 29 gave multiple radioactive peaks and was not further studied. High pressure liquid chromatography on reverse phase supports was also tried, but phos-

TABLE I

AMINO ACID COMPOSITIONS OF PHOSPHOLAMBAN AND CALCIDUCTIN RESIDUES PER 100 RESIDUES

n.d., not determined. (a) purified by HPLC on silica gel column. Mean of three determinations using three different sarcoplasmic reticulum preparations after 24 h hydrolysis; Thr and Ser not corrected ($X \pm$ S.D. of series; $n = 3$); (b) as given in Ref. 13; (c) as given in Ref. 30; (d) as given in Ref. 31; (e) approximate values obtained from Ref. 36; (f) calciductin amino acid composition from Ref. 8.

	Phospholamban					Calciductin
	(a)	(b)	(c)	(d)	(e)	(f)
Asx	8.3 ± 0.1	11.1	10	8.93	9	6.8
Thr	5.1 ± 0.6	5.5	5.5	4.79	5	5.8
Ser	6.2 ± 1.0	5.5	9.5	5.75	10	5.8
Glx	10.0 ± 0.2	11.4	12.2	16.7	12.5	7.7
Pro	4.2 ± 0.6	5.5	3.3	2.34	2.5	5.8
Gly	7.9 ± 0.4	10.6	11.7	1.85	12	8.3
Ala	9.7 ± 0.3	11.0	8.33	8.55	7	9.7
Cys	n.d.	0	0	n.d.	1	0.4
Val	6.3 ± 0.06	6.5	4.44	5.08	5	6.7
Met	2.9 ± 0.3	1.7	2.1	5.90	1.8	3.65
Ile	5.8 ± 0.6	4.9	2.2	9.77	5.8	6.6
Leu	13.2 ± 0.9	9.5	6.7	13.3	9	13.4
Tyr	3.0 ± 0.6	2.1	2	2.29	4	3.0
Phe	5.3 ± 0.2	3.4	10.6	3.68	4	6.2
Lys	5.6 ± 0.1	4.9	6.1	2.51	5	4.05
His	1.6 ± 0.3	2.0	3.3	< 0.1	2	2.2
Arg	4.9 ± 0.6	4.4	2.8	8.63	5	3.9
Trp	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

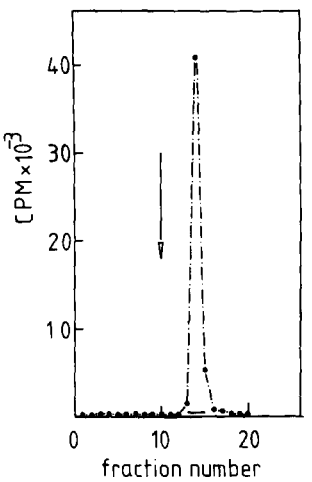

Fig. 1. Purification of [^{32}P]phosphate-labeled phospholamban by HPLC on a silica column. 4-min fractions were collected. The second solvent was started when indicated by the arrow (fraction 10). Fractions under the bar were pooled. Other conditions as described under Methods.

pholamban could not be recovered when the acidic chloroform/methanol extract was applied on an octadecyl silica column.

Previous studies have already shown that phospholamban could be separated from lipids by thin-layer chromatography on silica gel plates [26]. For preparative purposes, high pressure liquid chromatography on microparticulate silica was therefore tried. The acidic chloroform/methanol extract was loaded onto a silica gel column and the column was washed with chloroform/methanol/water/trifluoroacetic acid (200 : 100 : 0.75 : 0.3, v/v) to remove non covalently bound lipids (HCl was replaced by trifluoroacetic acid to avoid corrosive effects of chloride ions). Phospholamban was then eluted by a solvent of increased polarity chloroform/methanol/water/trifluoroacetic acid (30 : 30 : 10 : 0.07, v/v), where proteolipids are know to be soluble [28], as shown in Fig. 1. The amoun of phospholamban recovered, given by amino acid analysis was about 100 μg per ml of acidic chloroform/methanol extract loaded onto the column; this would correspond to an overall yield of 3.37 mg starting from 100 mg of sarcoplasmic reticulum proteins. Purified phospholamban showed one main band when run on a 15% (w/v) polyacrylamide slab gel in presence of SDS (Fig. 2). The main band corresponded to M_r about

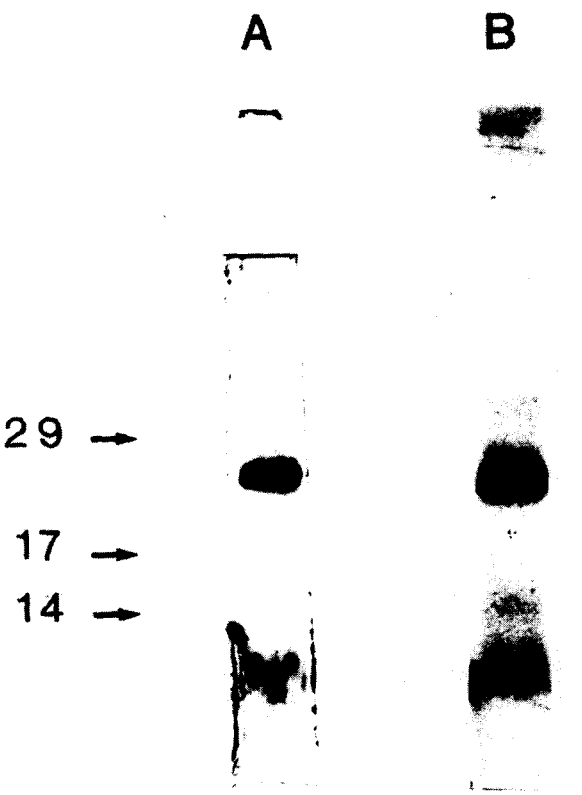

Fig. 2. Polyacrylamide gel electrophoresis of [^{32}]phosphate-labeled phospholamban purified by HPLC. The gel was stained with Coomassie blue (pane A) and autoradiographed (panel B). The figures referred to molecular weight markers ($\times 10^{-3}$): carbonic anhydrase (29), β-lactoglobulin (17) and lysozyme (14).

25 000, close to the usually obtained M_r 22 000, when electrophoresis was carried out in the absence of non ionic detergent [3,13,26]. In contrast, to previous results [13], phospholamban appeared to be normally stained by Coomassie blue. Tightly bound lipids, which could prevent stain binding, and are more efficiently removed by chromatography, might explain this difference. A faint and diffuse band of M_r about 11 000 was also detected by Coomassie blue staining and autoradiography, that probably corresponds to the monomeric form of phospholamban, which was obtained after treatment of the M_r 22 000 form by non-ionic detergent [13]. Phospholamban subunits might well be more tightly associated when freed of lipids, through association of hydrophobic domains in the protein subunits.

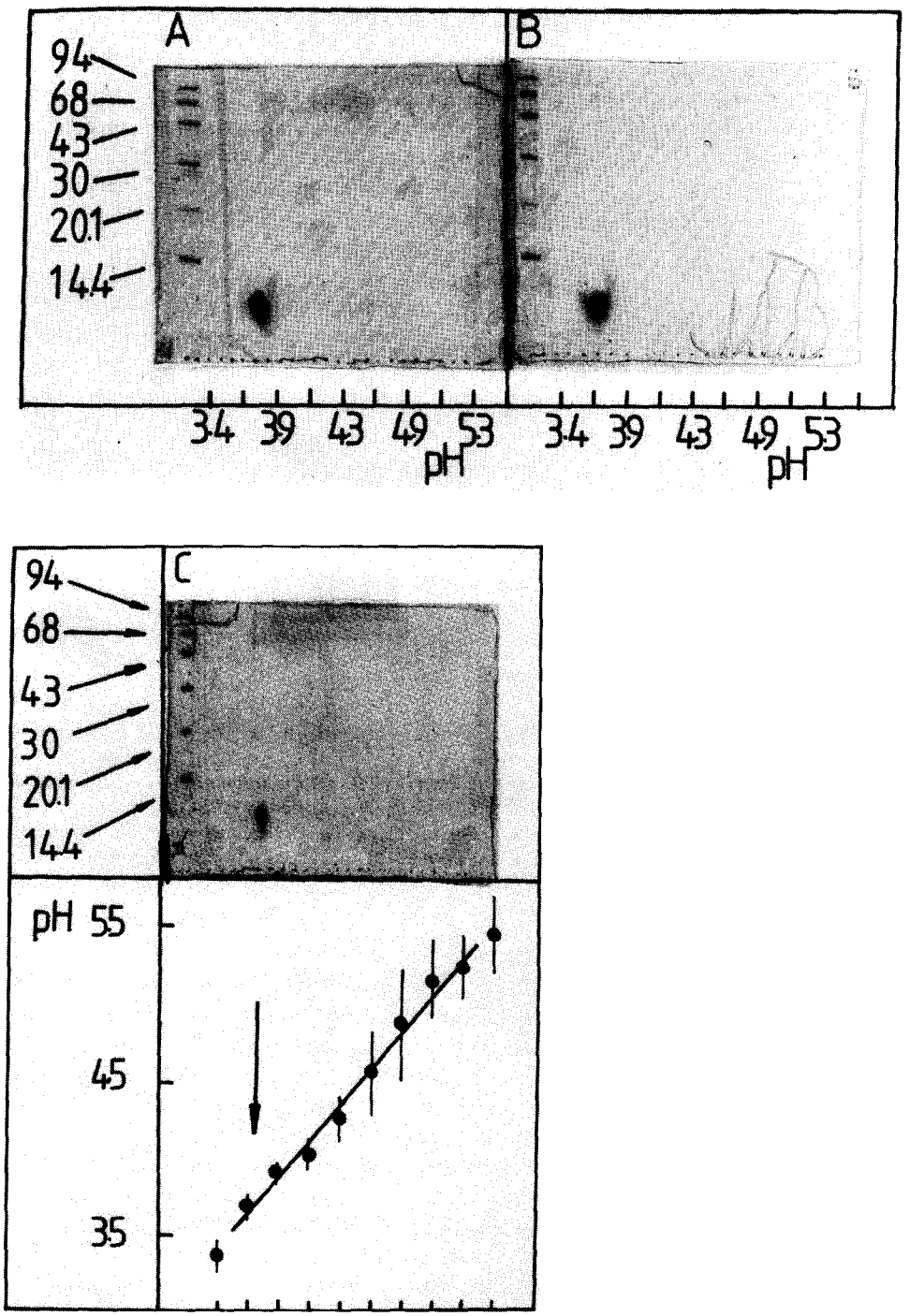

Fig. 3. Autoradiograms of two-dimensional gel electrophoretograms of acidic chloroform-methanol extracts [^{32}P]phosphate-labeled: (A) sarcoplasmic reticulum, (B) sarcolemma, (C) mixture of sarcoplasmic reticulum and sarcolemma extracts. First dimension: isoelectric focusing with pH values indicated on the horizontal axis. Second dimension: 0.1% SDS-15% polyacrylamide slab gels with standard $M_r \times 10^{-3}$ proteins reported on the vertical axis (glycogen phosphorylase, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and lysozyme, in the order of decreasing M_r). Lower curve: pH values obtained from five isoelectric focusing gels.

The amino acid composition of phospholamban is depicted in Table I. As expected from its acidic isoelectric point, there is an excess of acidic residues over basic ones (18.3% against 12.1%); large amounts of hydrophobic residues (approx. 50%) are also found reflecting the hydrophobic nature of this intrinsic membrane protein. The amino acid composition reported here, is also compatible with the minimum M_r of 5500 demonstrated recently [14] after vigorous treatment of cardiac microsomes by SDS. As shown in Table I, this amino acid composition is rather close to three other compositions already reported for phospholamban [13,30,36], but it differs significantly from another one, reported in Ref. 31, particularly on the Glx, Gly and His values. Calciductin, the major proteolipid of cardiac sarcolemma, isolated by HPLC on microparticulate silica [8], and phospholamban have also similar amino acid compositions.

When run in a liquid phase sequencer, phospholamban was not extracted from the cup during the organic solvent washes, but failed to release any phenylthiohydantoin, probably reflecting a blocked N terminus. Phospholamban was also found to be highly resistant to proteolytic degradation and was not digested by trypsin, chymotrypsin or thermolysin, eventhough digestions were carried out in presence of detergent. The absence of an accessible N terminal-end, and the resistance to proteolysis could both be due to hydrophobic aggregation of phospholamban into high molecular weight forms.

Phospholamban and calciductin exhibit identical behavior on microparticulate silica [8], and similar amino acid compositions (Table I). They were further compared by two-dimensional gel electrophoresis as shown in Fig. 3: identical values of isoelectric points, $pI = 3.7 \pm 0.3$ ($X \pm$ S.D.; $n = 5$) and molecular weights, $M_r = 10\,900 \pm 1\,050$ ($X \pm$ S.D.; $n = 5$) were obtained. Furthermore a single radioactive spot was present when phospholamban and calciductin were coelectrophoresed on the same gel.

Discussion

Proteolipids are integral membrane proteins, soluble in organic solvents but insoluble in water, which are particularly abundant in brain white matter [32]. They have been purified by chloroform/methanol (2 : 1, v/v) extraction followed by dialysis to remove non-covalently bound lipids, by chromatography on silicic acid with various solvents [33], by reverse-phase high pressure liquid chromatography [34], and by gel filtration in organic media [35]. Phospholamban, the major phosphorylatable proteolipid from sarcoplasmic reticulum has been purified by preparative gel electrophoresis [13,36], by extraction in the presence of very low amounts of deoxycholate followed by gel filtration in aqueous buffers [30], or by gel filtration in organic solvents after extraction of microsomes in aqueous buffers containing SDS [31]. We have found that phospholamban can be purified by high pressure liquid chromatography on microparticulate silica, after extraction from sarcoplasmic reticulum by an organic solvent mixture. The HPLC column is first washed for the removal of non covalently bound lipids and phospholamban is eluted by the front of the second solvent. The present purification method is fast and convenient, gives high recoveries and should be applicable to other acidic proteolipids as well. In contrast to one of the purification procedures previously described [30], phospholamban is obtained in its phosphorylated form, which is of primary importance for the demonstration of the presence of two different (a cAMP- and a calcium/calmodulin-dependent) phosphorylation sites. The yield is also much higher than in previous purification methods since 2.7 mg of phospholamban are obtained, compared with 400 and 235 μg, respectively, in Ref. 13 and Ref. 30, starting from 80 mg of sarcoplasmic proteins in all three cases.

The amino acid compositions of phospholamban isolated by five different methods do not differ widely, the largest differences being found for the Ser, Glx, Pro, Gly, Met, Phe, His and Arg values. The very low value of His ($< 0.1\%$) found in one of these methods [31], based on gel filtration in organic solvent mixtures, was claimed to be an indication of homogeneity of this preparation. In our hands, purified phospholamban, when run on an LH60 column in acidic chloroform/methanol (2 : 1, v/v), was eluted with a K_{av} of about 0.25, much lower than the value deduced from

Ref. 31, and without any change in the amino acid composition of the recovered peak. All these discrepancies would be only solved by the determination of the amino acid sequence of phospholamban. It was reported recently [14] that the monomeric form of phospholamban correspond to an M_r of about 5500 and that the 11000 [13] and the 22000–24000 M_r forms [3] previously isolated were dimers and tetramers, the lowest molecular weight form being obtained only after boiling in SDS prior to gel electrophoresis. The amino acid composition reported here is compatible with such a low molecular weight. Phospholamban purified by HPLC on microparticulate silica is obtained in high yield, about 250 nmol of M_r 11000 from 80 mg of sarcoplasmic reticulum protein. If phospholamban were present in the membrane in a one-to-one association with the calcium pump as described in Ref. 13, the overall yield would be about 40%, much higher than the 6% yield obtained using the diagonal procedure [13]. Phospholamban is therefore a major component of sarcoplasmic reticulum and accounts for about 3% (w/w) of sarcoplasmic proteins.

Calciductin is the major proteolipid of heart sarcolemma. It is phosphorylated by a cAMP-dependent protein kinase, and represents about 2% (w/w) of sarcolemmal proteins. Similarities between phosphorylatable proteolipids from cardiac sarcoplasmic reticulum and cardiac sarcolemma have already been documented [10,11]: identical molecular weights (M_r about 23000) were obtained in gel electrophoresis. We have further extended the comparison between phospholamban and calciductin and shown that they have similar behavior on microparticulate silica, similar amino acid compositions and identical molecular weights and isoelectric points. Both are also phosphorylated on seryl residues [8,13,24]. Whether calciductin and phospholamban are closely related or identical proteolipids might be proven only by the determination of their primary structure, but cross-contamination of the two membrane preparations can be ruled out, since sarcolemmal vesicles were shown to contain less than 4% of sarcoplasmic reticulum vesicles [8].

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