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Biochimica et Biophysica Acta 1417 (1999) 1–8

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

A novel small protein associated with a conjugated trienoic chromophore from membranes of scallop adductor muscle: phosphorylation by protein kinase A

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Received 1 September 1998; received in revised form 16 November 1998; accepted 20 November 1998

Abstract

Membranes enriched in sarcolemma from the cross-striated adductor muscle of the deep sea scallop have been found to contain a previously undescribed small protein of 6–8 kDa that can be released by treatment with organic solvent mixtures. This proteolipid co-purified with a non-amino acid chromophore containing a conjugated trienoic moiety. Although common in plants and algae, such a stable conjugated trienoic group is unusual for an animal cell. The N-terminal amino acid sequence of the protein was XEFQHGLFGXF/ADNIGLQ, which most strongly resembles sequences in the triacyl glycerol lipase precursor and the product of the human breast cancer susceptibility gene BRCA 1, but does not show similarity to previously described proteolipids. The protein was found to be one of the major substrates in its parent membrane for the catalytic subunit of protein kinase A, which may imply a regulatory function for this molecule.

Keywords: Proteolipid; Triene; Sarcolemma; Scallop; Protein kinase A

1. Introduction

Proteolipids are operationally defined as proteins soluble in organic solvents [1,2]. Small proteolipids with an amphipathic organization have been found in muscle. These include sarcolipin and phospholamban of the sarcoplasmic reticulum, which are involved in regulation of the SERCA1 and 2a isoforms of the Ca^{2+}-ATPase of the sarcoplasmic reticulum [3–6]. Phospholemman, found in cardiac sarcolemma [7], has similarities to the proteolipid γ-subunit of the Na^+,K^+-ATPase (e.g. [8–10]), which may regulate certain steps of the pumping cycle of that enzyme [11,12].

The cross-striated (phasic) part of scallop adductor muscle has been the subject of many biochemical, physiological, and structural investigations. Thus, very extensive studies have been made of myosin-linked regulation in the adductor muscle (e.g. [13]); and the Ca^{2+}-transporting ATPase of the sarcoplasmic reticulum (SR) membrane has also been examined (e.g. [14,15]). Foot-type structures probably related to ryanodine Ca^{2+}-release channels have been

Abbreviations: DITC, 1, 4-phenylene diisothiocyanate; FSR, fragmented sarcoplasmic reticulum; MOPS, 3-(*N*-morphilino)-propanesulfonic acid; PK-A, protein kinase A (cAMP-dependent protein kinase); SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; SL, sarcolemma; SR, sarcoplasmic reticulum

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0005-2736/99/$ – see front matter
PII: S0005-2736(98)00241-7

observed in scallop muscle membrane preparations [16]. However, the biochemistry of the sarcolemma of the scallop muscle cell remains essentially undescribed. In a preliminary study of scallop sarcolemma, we have observed that preparations enriched in that membrane possess a very distinctive near UV spectrum typical of a conjugated triene moiety, and that this is associated with a small protein extractable in organic solvents. This protein is one of the major substrates in the membrane for protein kinase A (PK-A).

2. Materials and methods

Deep sea scallops (*Placopecten magellanicus*) were obtained from the Marine Biology Laboratory, Woods Hole, MA. All chemicals were ACS grade or better.

2.1. FSR, enriched SL, and deoxycholate-extracted SR

The cross-striated part of the adductor muscle of the deep sea scallop was first dissected and freed of any other tissue, and the membrane fractions then prepared from it essentially as described previously [17,18].

2.2. Preparation of the proteolipid

The procedure initially followed that for the preparation of scallop adductor muscle membrane fractions as described previously [17,18]. The material banding at the 0.32–0.8 M sucrose interface was collected, and washed free of sucrose into a solution of 50 mM ammonium acetate, pH 6.8. Twenty volumes of $CHCl_3$–MeOH, 2:1, v/v, were then added to yield a single phase. The preparation was centrifuged at $10^4 \times g$ for 1 h, the supernatant carefully collected, and filtered under vacuum through three layers of Whatman no. 1 filter paper on a fine glass sinter. Three volumes of 50 mM ammonium acetate, pH 6.8, were then added to give two phases, and the emulsion was centrifuged at $10^4 \times g$ for 1 h. The aqueous/MeOH upper layer containing the proteolipid was collected and subjected to rotary evaporation to remove MeOH, after which the solution was diluted 2-fold with water and freeze-dried. (The proteolipid could also be precipitated from the initial $CHCl_3$–MeOH extract with 5 vol. ice-cold diethyl ether, and collected by centrifugation at 1–$2000 \times g$ in glass tubes.) The freeze-dried material was dissolved in 50 mM ammonium acetate, pH 6.8, and passed through a 1×8 cm column of Sephadex G-50 (fine) in the same solvent to remove contaminating water-soluble small molecules, such as sucrose, when it emerged in the void volume (probably in the form of large mixed micelles with glycolipids and lysophospholipids that also partition into the aqueous phase). To further purify the proteolipid from glycolipids, three volumes of ice-cold acetone were then added, and after 30 min at −20°C to allow the precipitate to coagulate, the preparation was centrifuged at $2000 \times g$ at 4°C for 5 min. The pellet was washed with ice-cold acetone and dried in a gentle stream of argon. The dried acetone precipitate was dissolved in water, and extracted twice with 3 volumes of water-saturated 1-butanol. The lower aqueous phase contained the conjugated trienoic chromophore and proteolipid. Final purification was by TLC on silicic acid using as mobile phase 1-propanol–H_2O, 7:3, v/v (when the proteolipid has an R_f of 0.47), or $CHCl_3$–MeOH–H_2O, 5:7:1, v/v/v (when the proteolipid remains at the origin.). The molecule was eluted from the scraped silica with $CHCl_3$–MeOH–2.5 M NH_4OH, 60:40:9, v/v/v. The purified material was stored dry under argon at −80°C.

2.3. Electrophoresis

Polyacrylamide gel electrophoresis in SDS used the Tris-Tricine system [19].

2.4. Protein determinations

This was by the bicinchoninic acid method [20].

2.5. Carbohydrate determination

Neutral hexose was assayed by the phenol-sulfuric acid method [21]. Bial's reagent (0.9% w/v $FeCl_3$, 0.55% w/v orcinol in acidified ethanol) was used as a spray reagent for qualitative detection of sugar on TLC.

2.6. Organic phosphorus

This was determined by the method of Bartlett [22].

2.7. Total cholesterol (cholesterol and cholesterol esters)

This was determined by a coupled enzyme procedure using cholesterol esterase, cholesterol oxidase and peroxidase [23].

2.8. N-terminal amino acid sequencing

One proteolipid preparation was adsorbed onto Immobilon-P (Millipore), and sent to the Protein Chemistry Laboratory, Washington University. A second sample was reacted with Sequelon DITC- and arylamine-derivatized PVDF discs, essentially according to the protocols supplied by PerSeptive Biosystems. Sequencing of the samples bound through amino groups to the DITC membranes, and through carboxyl groups to the arylamine membranes was carried out simultaneously, to allow complementary compensation for loss of coupled residues to the two different types of membrane. N-Terminal sequencing of this sample was carried out at the University of Florida Protein Core.

2.9. Treatment with protein kinase A

SL and SR (0.4 mg) were incubated at 23°C for 1.5 min in a medium of 0.32 M sucrose, 0.1 M KCl, 0.1 mM [γ-^{32}P]ATP (500 cpm pmol^{-1}), 2 mM DTT, 35 mM NaF, 5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 5 mM EGTA, 20 mM MOPSNa, pH 7.0 in a volume of 120 μl with 18 U of the catalytic subunit of PK-A (Sigma). The phosphorylation was stopped by adding 2×Tricine gel sample denaturation buffer. Gels were run in the Tricine system, and dried onto Whatman no. 1 filter paper, and autoradiography carried out.

2.10. Ca^{2+}-ATPase activity

This was measured as described previously [17].

2.11. Na^+,K^+-ATPase activity

This was measured as described previously [24].

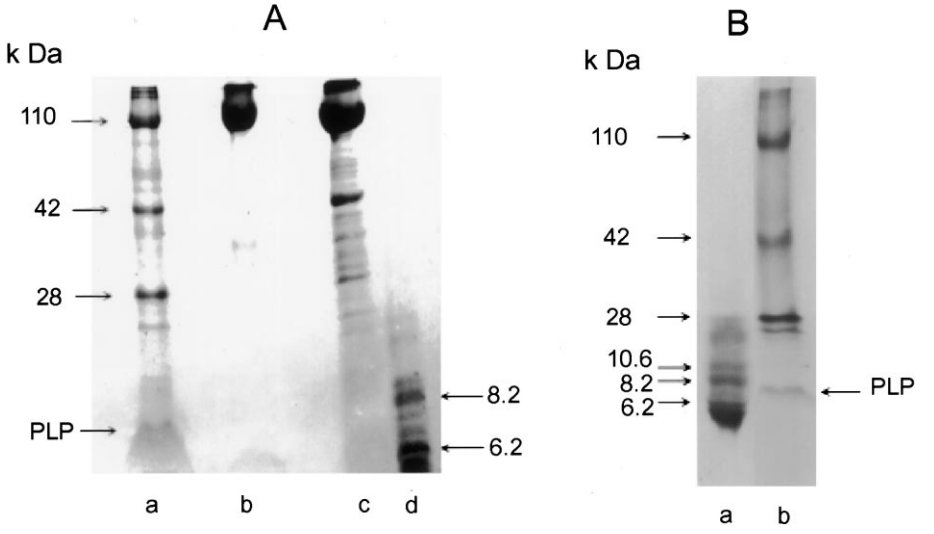

Fig. 1. Polypeptide composition of the B_1 and FSR membrane fractions. (A) Tricine SDS-PAGE with a 5% stacking gel and 10% separating gel, stained with Coomassie brilliant blue R-250. Lane a, B_1 fraction (enriched SL) (15 μg); lane b, deoxycholate-extracted SR (membranous Ca-ATPase) (40 μg); lane c, FSR (50 μg); lane d, cyanogen bromide fragments of myoglobin, and glucagon (Sigma) (4 μg). PLP, proteolipid. (B) Tricine SDS-PAGE using a 5% stacking gel, 10% intermediate gel, and 16.5% separating gel. Lane a, myoglobin fragments (Sigma); lane b, enriched SL (21 μg). PLP, proteolipid.

3. Results

3.1. Preliminary characterization of a membrane fraction enriched in sarcolemma

The membrane fraction (B_1) used subsequently to prepare the proteolipid was collected from the 0.32–0.8 M sucrose interface. It had a greater ouabain-inhibitable Na^+,K^+-ATPase activity, lower Ca^{2+}-ATPase activity and higher cholesterol content than the denser FSR fraction (Table 1). Comparison of SDS gels of the B_1 and FSR membrane fractions (Fig. 1A) indicated that the 110-kDa band of the Ca^{2+}-ATPase of the SR was decreased in intensity in the B_1 fraction relative to other components, in particular to the polypeptide of $M_r \sim 28$ kDa previously noted [17]. Examination of the B_1 fraction by negative staining in the electron microscope showed mainly sheet-like membranous structures (not shown), very different from the well-described vesicular scallop FSR [25,26]. Thin filaments were sometimes seen, and the band on SDS gels at 42 kDa is probably actin. Thus, the B_1 fraction is enriched in SL, but still contaminated by elements of the SR and thin filaments.

3.2. The enriched SL contains a 6–8-kDa protein and a conjugated trienoic chromophore

When the enriched SL fraction (B_1) was run on Tricine gels with only a 5% stacking gel and 10% separating gel, but no 16.5% layer, a relatively diffuse, irregular, distorted band with a mobility corresponding to 6–8 kDa could be seen (Fig. 1A, lane a). This was present in significantly smaller amounts in the FSR fraction (Fig. 1A, lane c), and is almost absent from the deoxycholate-extracted SR (purified membranous Ca^{2+}-ATPase) (Fig. 1A, lane b). On Tricine gels with an additional 16.5% separating layer, the 6–8 kDa band could be seen more clearly (Fig. 1B). The near UV spectrum of the enriched SL preparation displayed strong absorption peaks at 260, 271 and 282 nm (Fig. 2A), suggesting the presence of a conjugated triene [27].

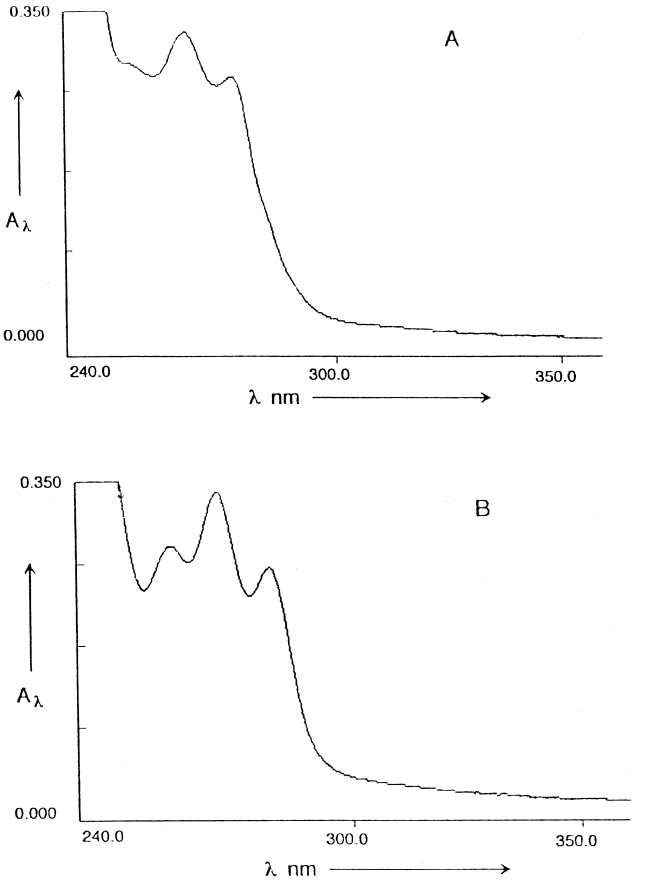

Fig. 2. Near UV spectra of the enriched sarcolemmal fraction and the proteolipid. (A) Enriched SL was prepared as described in Section 2 and dissolved in 1% w/v SDS, 20 mM MOPSNa, pH 7.0, to a final total protein concentration of 0.6 mg ml^{-1}. (B) Proteolipid dissolved in 1% w/v SDS, 20 mM MOPSNa, pH 7.0, to a final protein concentration of 0.03 mg ml^{-1}.

Table 1
Comparison of scallop muscle membrane fractions

	Enriched SL	FSR
Ca^{2+}-ATPase activity (μmol ATP min^{-1} mg $protein^{-1}$)	0.32 ± 0.1	3.2 ± 0.4
% of Ca^{2+}-ATPase sensitive to oligomycin	2 ± 1	13 ± 4
Ouabain-inhibitable Na^+,K^+-ATPase activity (μmol ATP min^{-1} mg $protein^{-1}$)	0.19 ± 0.03	0.09 ± 0.02
Organic phosphorus (μmol P mg $protein^{-1}$)	1.7 ± 0.1	1.10 ± 0.04
Cholesterol and cholesterol esters (μmol mg $protein^{-1}$)	0.9 ± 0.1	0.30 ± 0.04

Each value is the mean and standard deviation of four measurements.

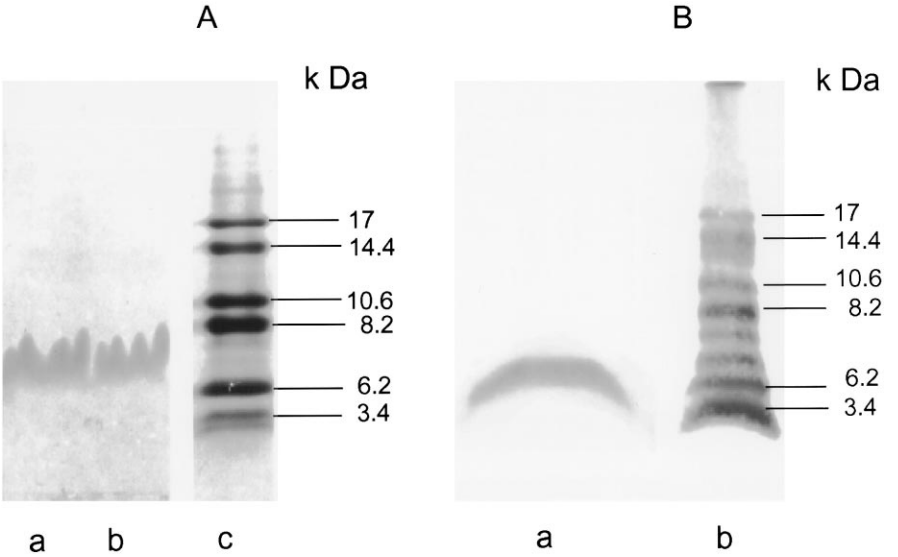

Fig. 3. Proteolipid prepared from a membrane fraction enriched in sarcolemma. Tricine SDS gels with 5% upper gel layer; 10% middle gel layer; 16.5% separating gel layer. (A) Aqueous phase from organic solvent extraction after freeze-drying. Lanes a and b, 3 μg proteolipid; lane c, myoglobin cyanogen bromide low molecular weight standards (Sigma). (B) Proteolipid after TLC purification. Lane a, 5 μg proteolipid; lane b, myoglobin cyanogen bromide low molecular weight standards (Sigma).

When the whole aqueous phase from the organic solvent extraction step was freeze-dried and dissolved in Tricine gel sample denaturation buffer, only a single, distorted, Coomassie blue-stained band was seen on SDS gels, with a mobility corresponding to 6–8 kDa (Fig. 3A). Thin layer chromatography on silicic acid with solvents of intermediate polarity (e.g. $CHCl_3$–MeOH–H_2O, 14:6:1, v/v/v) gave only a single spot at the origin that charred with 40% v/v H_2SO_4 on heating. However, TLC in solvents of higher polarity, such as 1-propanol–H_2O, 7:3, v/v or $CHCl_3$–MeOH–H_2O, 5:7:1, v/v/v, revealed at least seven other components besides the proteolipid. Thus, while the partitioning step very efficiently separated the proteolipid from the bulk of the lipid, further purification as described in Section 2 was necessary to remove more polar contaminants. (These non-proteolipid species were not investigated further in this work.) The proteolipid spot of R_f 0.47 (in 1-propanol–H_20, 7:3, v/v) was stained with I_2, consistent with an unsaturated grouping being associated with the protein.

After TLC purification, the proteolipid band on SDS gels was sharper (Fig. 3B). The near UV absorption spectra of the proteolipid resembled that of the B_1 membrane fraction, with three prominent absorption bands having maxima at 260, 270.8 and 282

Table 2
N-Terminal amino acid sequence of the proteolipid

Cycle	Sample 1		Sample 2	
	Residue	Yield (pmol)	Residue	Yield (pmol)
1	–	–	–	–
2	E	28	E	12.95
3	F	55	F	15.45
4	Q	36	Q	14.61
5	–	–	H	4.53
6	G	33	G	7.51
7	L	36	L	11.33
8	F	34	F	9.09
9	G	37	G	5.84
10	–	–	–	–
11	F	39	A/F	(1.02/0.62)
12	D	18	D	0.68
13	N	8	N	0.61
14	I	15	I	1.84
15	G	20	G	0.43
16	L	19	L	1.27
17	–	–	Q	0.80
18	–	–	(D)	0.20
19	–	–	A	0.90
20	–	–	I	0.42

Two separate samples of the proteolipid were prepared. One sample (1) was adsorbed onto Immobilon-P, and the other (sample 2) covalently reacted with DITC- and arylamine-derivatized disks (see Section 2).

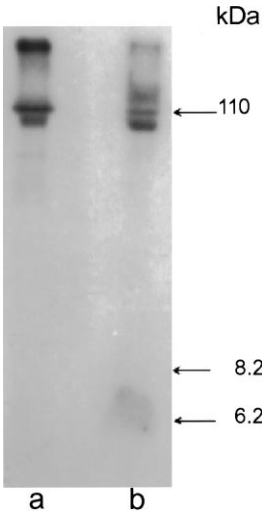

Fig. 4. Treatment of the SL and SR fractions with the catalytic subunit of PK-A. The phosphorylations were carried out as described in Section 2. Phosphorylated samples were run on Tricine SDS gels with a 5% upper gel and a 12% separating gel. Gels were dried onto filter paper and exposed to Kodak X-OMAT film. Lane a, FSR, 20 μg; lane b, enriched SL, 20 μg.

nm (Fig. 2B), typical of a conjugated triene [27]. This strongly absorbing chromophore obscures any absorption from the protein. Assuming an extinction coefficient of 5.7×10^4 M^{-1} cm^{-1} at 271 nm for the conjugated triene [28], and that the protein does not contribute significantly to the absorption spectrum, the molecule contained a ratio of 1.2 ± 0.4 mol of chromophore per 7 kDa protein (average of 4 measurements), suggesting a possible stoichiometric relationship between the chromophore and the small protein. One proteolipid preparation was adsorbed onto Immobilon-P PVDF membrane; and a separate preparation reacted with the DITC and arylamine derivatized disks, as described in Section 2. The N-terminal sequences from the two preparations shown in Table 2 are in good agreement, and the probable sequence is XEFQHGLFGXF/ADNIGLQ. Sugar was detected qualitatively in the proteolipid band on TLC by spraying with Bial's reagent. When the phenol-sulfuric acid carbohydrate assay was calibrated with an equimolar mixture of D-mannose and D-galactose, it was found that there were 3.3 mol neutral hexose per mol 7 kDa protein (average of three measurements). The material displayed some unusual solubility properties. Very extensive washing of the starting muscle membrane preparation with aqueous media expected to extract peripheral membrane proteins (e.g. 1 M KCl, 10 mM EDTA, 50 mM Na borate, pH 9.8) did not remove the protein, but once released from the membrane, the molecule could be dispersed in water, or dissolved in $CHCl_3$–MeOH–2.5 M NH_4OH, 60:40:9, v/v/v. The chromophore and protein were coprecipitated from water by the addition of ammonium sulfate to 60% saturation (4°C).

3.3. Phosphorylation of the proteolipid by protein kinase A

Treatment of the membrane fraction enriched in SL (B_1) and the FSR fraction with the catalytic subunit of PK-A and [γ-P^{32}]ATP led to the phosphorylation of a restricted number of proteins, as judged by autoradiography of Tricine SDS gels (Fig. 4). Three proteins were strongly phosphorylated in the enriched SL: the 110-kDa band, a 95-kDa band, and the proteolipid. (An estimation of ^{32}P incorporation into protein partitioning into the aqueous phase after chloroform–methanol extraction gave 0.4 mol per 7000 g protein.)

4. Discussion

Many otherwise highly unsaturated biological lipids do not show conjugation amongst the double bonds, which are usually separated by one methylene group, although double bonds further apart can occur, e.g. in non-methylene-interrupted dienoic fatty acids. Nevertheless, there are numerous reports of stable conjugated trienes in plants and algae (e.g. [29]). The eicosanoids and retinoids provide examples of molecules with conjugated double bonds in animals. The retinoids are conjugated pentaenes, further conjugated to carbonyl groups in retinal and retinoic acid, and these compounds display electronic transitions in the visible part of the spectrum, at longer wavelengths than any absorption peak observed with the scallop chromophore. Carotenoids, dietary precursors of the retinoids, show even more extensive conjugation. In the case of eicosanoids, the lipoxygenase pathways, in particular, give rise to many molecules containing conjugated double bonds (e.g. [27]), such as hydroperoxy- and hydroxyeicosatetraenoic acids (HPETE and HETE). The lipoxins (see [30]),

produced by the action of more than one lipoxygenase, have conjugated tetraene groups. The conjugated trienoic grouping itself is present in the leukotrienes, paracrine and autocrine mediators of inflammation and smooth muscle contraction produced by the lipoxygenase pathway in a variety of immune cells. While polyunsaturated fatty acids including 20:3, 20:4, 20:5 and 22:6 have been found in marine bivalves, including the scallop [31], conjugation amongst the double bonds has not been reported. Since the muscles used in the preparation of the proteolipid were dissected out, cleaned, and the sarcolemmal-enriched membrane fraction isolated before the $CHCl_3$–MeOH extraction step, it is unlikely that the trienoic chromophore reported here is derived from any direct algal contamination of the tissue used as starting material. The chemical nature, physiological role and biosynthetic origin of the chromophore are of interest for further investigation. The molecule to which the conjugated triene moiety belongs may have a structural function, e.g. as a non-polar anchor for the protein in the sarcolemmal membrane. The deep sea scallop lives at a low ambient temperature [17], and the presence of the unsaturated group may reflect the need to maintain membrane fluidity under such extreme conditions. The conjugated grouping may be synthesized by the scallop itself, or originate in the diet, possibly in algae, some of which are known to possess an isomerase able to convert unconjugated fatty acids into conjugated trienes [29,32].

The solubility properties of the proteolipid are unusual, in that it apparently dissolves in both water and, for example, $CHCl_3$–MeOH–2.5 M NH_4OH, 60:40:9, v/v/v. However, whether it forms a truly monomeric solution in water, or is in the form of a micellar-like state remains to be investigated. Lipophilin, the proteolipid of myelin which is ordinarily insoluble in water, can be transferred from organic solvents, such as 2-chloroethanol, into aqueous media by dialysis procedures [33]. Prior to TLC purification, the detergent-like nature of contaminating glycolipids and lysophospholipids also extracted into the aqueous phase may enhance dispersion of the proteolipid in aqueous media.

The subcellular location of the scallop muscle proteolipid remains to be determined more precisely. Although the B_1 membrane fraction used for the preparation of the proteolipid was enriched in SL, it still had some Ca^{2+}-ATPase activity, and therefore must be contaminated by elements of the SR. In turn, the FSR fraction has Na^+,K^+-ATPase activity, and so must contain some membranes derived from the SL. Nevertheless, since scallop FSR consistently contained significantly less of the proteolipid, the proteolipid is probably associated with the SL component of the B_1 fraction, or some specialized region of the SR involved in making contact with the SL that remains bound to fragments of that membrane after disruption of the muscle. Although the scallop muscle lacks transverse tubules, it has junctions between the SL and cisternae of the underlying SR which resemble the contacts between the transverse tubules and terminal cisternae of vertebrate skeletal muscle SR [34], and ryanodine type Ca^{2+} release channels have been found [16]. Presumably, the patches of SL of the scallop cross-striated muscle cell overlying these junctions fulfil some of the functions of transverse tubules of vertebrate cross-striated cells. Transverse tubules of vertebrate cross-striated muscle membranes possess a 28-kDa protein [35], and the presence of a prominent 28-kDa band on SDS gels of the scallop B_1 membrane fraction raises the possibility that the latter may contain structures derived from the junctional region between the SL and SR. Future studies will require detailed subfractionation and investigation of the enriched SL preparation. The presence of carbohydrate implies that at least part of the proteolipid faces the external medium, or an internal lumen.

Comparison of the N-terminal amino acid sequence of the scallop proteolipid with known sequences using the EMBL data base ('Blitz'), indicated a modest similarity to residues 15–24 of the triacylglycerol lipase precursor (46% match), and to residues 1226–1240 of the human breast cancer susceptibility gene product BRCA 1 (43% match), but it did not match sequences from any of the previously described proteolipids. The fact that the proteolipid can be phosphorylated in situ in its parent membrane by PK-A indicates that at least the part of the proteolipid polypeptide chain containing a phosphorylatable serine/threonine is exposed on one of the surfaces of the parent membrane, and accessible to the kinase. Phosphorylation of phospholamban by PK-A mediates the effect of cAMP on the SERCA2a iso-

form of SR Ca^{2+}-ATPase (e.g. [6]), and the function of that proteolipid is well understood. Recent studies indicate a possible role for sarcolipin as a physiological regulator of SERCA1, the isoform of the Ca^{2+}-ATPase expressed in skeletal muscle [5]. Two isoproteolipids may modulate the H^+-ATPase activity of the plasma membrane of *Saccharomyces cerevisiae* [36,37]. The physiological role of the relatively well-studied γ-subunit of the Na^+,K^+-ATPase, has not been clear [10,24]; but recent studies suggest that it may modulate the K^+-activation of the Na^+,K^+-ATPase [11], and be involved in the occlusion of K^+ by that enzyme [12]. The function of phospholemman, a proteolipid present in cardiac sarcolemma [7] which has a 52% amino acid sequence identity with the γ-subunit of the Na^+,K^+-ATPase, remains unclear. However, it is known to be a major substrate for PK-A, and so, like the γ-subunit, phospholamban, sarcolipin and the H^+-ATPase proteolipids, may form part of a regulatory mechanism. Like phospholemman, the function of the proteolipid from scallop muscle is at present unknown, but may be regulatory.

Acknowledgements

This work was supported by the Office of Research and Development, Southern Illinois University; and a Central Research Committee award from the School of Medicine, Southern Illinois University.

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