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CHEMICAL COMPOSITION OF SARCOLEMMA ISOLATED FROM RABBIT SKELETAL MUSCLE

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SUMMARY

1. Sarcolemma isolated from rabbit skeletal muscle is composed of 63% protein, 17.5% lipid, 3–5% nucleic acids and a trace amount of carbohydrate. The lipid fraction is composed of 50% neutral lipid and 50% phospholipid.

2. The major classes of neutral lipids and phospholipids were determined. As neutral lipids we found cholesterol and cholesterol esters. As phospholipids were found lecithin (43%), phosphatidylethanolamine (24%), sphingomyelin (23%) and lysolecithin (10%).

3. By means of sodium dodecyl sulfate–polyacrylamide gel electrophoresis it was possible to separate twelve main protein bands whose molecular weights range from 12000 to 200 000.

INTRODUCTION

The most important biochemical properties of the biological excitable membranes are related to their ability to control the permeability to ions[1,2] such as Na^+ and K^+. It seems that permeability control is exerted by means of interactions of cations such as Ca^{2+} and others with active sites existing in the membranes which are responsible for the permeability regulation[2]. Thus, it is of importance to investigate the membrane components that may act as permeability regulators.

Phospholipids are the membrane components interacting with cations which are probably implicated in permeability regulation and ion transport[3–5]. Removal of membrane phospholipids has the effect of decreasing the binding of cations by sarcolemma[6] and other cell membrane systems[5,7,8]. Although in the case of the sarcolemma, the phospholipids represent only about 8.5% of the total dry weight, removal of this membrane component greatly decreases the binding of cations by the remaining sarcolemmal structure. Therefore, it is reasonable to think that phospholipids are the major chemical components of sarcolemma that bind cations, which suggests that the phospholipids may be important in controlling the permeability and other biological parameters of the muscle membrane.

In a previous paper we studied the interaction of cations and local anesthetics with isolated sarcolemma[6] and we showed that the anesthetics compete for the same sites which bind Ca^{2+}. In the present work we study the chemical composition of the sarcolemma as a preliminary approach to determine in further studies the com-

ponents of the muscle membrane involved in its interaction with Ca^{2+} and local anesthetics.

Furthermore, it would be of interest to determine the membrane components involved in the particular Ca^{2+}-ATPase activity of the sarcolemma[9–13]. This activity is probably associated with the efflux of Ca^{2+} from the muscle cell against its chemical gradient[9–12], an event which may play an important role in the excitation–contraction coupling of the muscle cell. Although some effort has been made to determine the membrane components of sarcolemma involved in its enzymatic activity[14], no relevant information is available at present. Thus, in further studies we will attempt to approach the problem, starting from the general information of the present work.

MATERIALS AND METHODS

Preparation of the biological material

Sarcolemma was isolated from the back and limb muscles of rabbit as described elsewhere[6,15,16]. The purified sarcolemma was homogenized in a glass–teflon homogenizer in order to obtain a fine suspension. The resulting membrane fragments were washed exhaustively with water to remove any contaminant materials such as actomyosin, fragmented sarcoplasmic reticulum and mitochondria. The preparations appeared homogeneous and no contaminant materials could be detected by electron microscopy (Fig. 1). The structures observed by us with the aid of the electron and phase contrast microscopy are similar to those observed by other authors[16–19].

General chemical composition of the sarcolemma

The protein content of membranes was estimated by the biuret method[20] in sarcolemmal suspensions solubilized in 4 M NaOH.

The procedure employed to extract the lipids and nucleic acids was that of Schneider[21] as modified by Carvalho and Leo[22]. The determination of phospholipid and nucleic acids was made in digested extracts in which the inorganic phosphate was determined by the method of Chen *et al.*[23]. The amount of inorganic phosphate was multiplied by a factor of 25 and 10.6 for the calculation of the phospholipid[24] and nucleic acid fraction[25], respectively, in the original samples. Furthermore, we estimated the DNA content by the diphenylamine method[26] and RNA by the $FeCl_3$–HCl–orcinol method[26] in the extracts containing the nucleic acids, using commercial DNA or RNA as standards.

Separation and determination of neutral lipids and phospholipids

For the determination of the various classes of lipids, we extracted the lipid fraction by the method of Folch *et al.*[28]. The extracts were dried under nitrogen and finally dissolved in a small volume of chloroform–methanol (2:1, v/v). Aliquots of this solution were spotted on glass plates (10 cm × 10 cm) covered with a layer of silica gel G (Merck), 0.5 mm in thickness. For the separation of neutral lipids into classes we ran the plates with *n*-hexane–diethyl ether–acetic acid (80:20:1, v/v/v) for about 10 min[29]. The plates were dried and the separated spots were developed under iodine vapours. For the separation of phospholipids into classes we employed the same technique described above using another solvent[30] containing chloroform–methanol–acetic acid–water (25:15:4:2, by vol.). For identification of the various

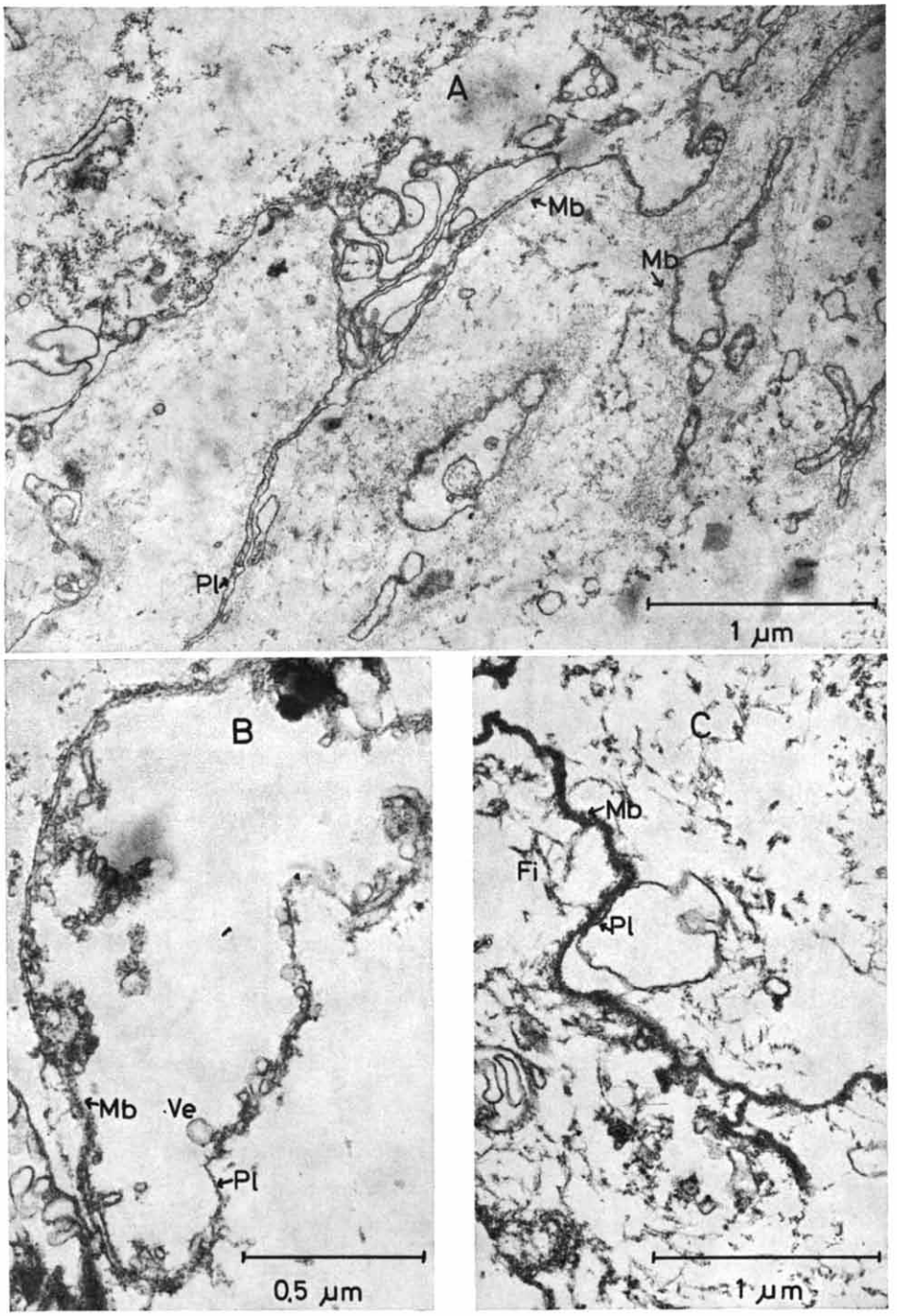

Fig. 1. Electron micrographs of isolated sarcolemma. Sarcolemma tubules were fixed in 1.25% glutaraldehyde, postfixed in 1% O_8O_4 with 10 mM $CaCl_2$, treated with 0.5% uranyl acetate and embedded in Epon. (A) Transverse section showing the plasma membrane (Pl), and punctuation marks resulting from the filaments which form the basement membrane (Mb). (B) Another transverse section showing vesicles (Ve) associated with the plasma membrane. (C) Longitudinal section of sarcolemma tubules showing the basement membrane (Mb) and the plasma membrane (Pl). Note the presence of filaments (Fi) which form the external network of the sarcolemma.

components, lipid standards obtained from Supelco, Inc. (Bellefonte, Pa.) were run simultaneously with the samples. For the quantitative determination of each phospholipid the spots were sucked from the plates directly into Kjeldahl flasks and the samples were digested[22]. Blank zones were taken out and digested in the same way. The silica gel was separated by centrifugation, the digested samples were analysed for inorganic phosphate[23] and the phosphate content of blanks was discounted in samples. Simultaneously phospolipid standards were also digested and the inorganic phosphate determined. Then it was possible to calculate the amount of each phospholipid by relating its amount in phosphate to the amount of phosphate in standards.

Separation of proteins and determination of molecular weights

The sarcolemmal fragments were dissolved in 10 mM phosphate (pH 7.0), containing 1% sodium dodecyl sulfate and 1% β-mercaptoethanol, for 1 h at 37 °C. The protein solution was dialysed for 24 h against 10 mM phosphate (pH 7.0), containing 0.1% sodium dodecyl sulfate and 0.1% β mercaptoethanol. Aliquots (50–100 μg) were submitted to polyacrylamide gel electrophoresis (10% acrylamide and 0.135% methylene bisacrylamide) by the method of Weber and Osborn[31], applying a current of 8 mA per tube during 5–6 h. The gels were stained with Coomassie blue for 15 h and destained electrophoretically for 5–6 h at 5 mA per tube. Simultaneously and using the same treatment as for samples, we ran standard proteins of known molecular weights. The relative mobility of the standard proteins in the gels was plotted against the log of their molecular weights and the molecular weights of the sample bands were determined. Furthermore, the gels were scanned in a Gelman scanning system and the absorbance of each band at 550 nm was recorded continuously in a Vitatron UR 405 recorder. The resulting peaks were integrated and, assuming that the concentration of protein is approximately proportional to the colour intensity of the bands, we could estimate approximately the relative amount of each band.

Reagents

All reagents used were of reagent grade for biochemical analyses. The phospholipid standards obtained from Supelco, Inc. (Bellefonte, Pa.) were egg lysolecithin (No. 6003), egg lecithin (No. 6000), egg phosphatidylethanolamine (No. 6002), bovine phosphatidylserine (No. 6004) and bovine sphingomyelin (No. 6009). The proteins used as standards in polyacrylamide gel electrophoresis were obtained from Sigma except myosin and calsequestrin which were isolated from rabbit skeletal muscle by standard procedures[32,33].

RESULTS

General chemical composition of the sarcolemma

The results (Table I) suggest that sarcolemma is composed of 63% protein, 17.5% lipid, 3–5% nucleic acids and a trace amount of carbohydrate. The lipid fraction is composed of about 50% neutral lipid and 50% phospholipid. There is a discrepancy in the estimated values for nucleic acids as determined by different methods (3 or 5%). The difference may result from errors inherent in the methods themselves or from the contribution of some nucleotide fraction present in the digested

TABLE I

CHEMICAL COMPOSITION OF SARCOLEMMA ISOLATED FROM RABBIT SKELETAL MUSCLE

Material	*% dry weight*	*μg/mg protein*
Protein (biuret)	63.2±3.0 (6)	
Phospholipid	8.6±0.5 (6)	137
Neutral lipid	8.8±0.6 (6)	
Nucleic acids (by inorganic phosphate)	5.3±1.0 (6)	82
DNA (diphenylamine)	2.3±0.3 (6)	37
RNA ($FeCl_3$-HCl-orcinol)	0.7±0.2 (6)	12
Carbohydrate (as glucose by anthrone)	0.7±0.2 (6)	12
Material not recovered	10.4	

extracts whose inorganic phosphate was analysed together with that of nucleic acids.

Separation of neutral lipids

The neutral lipid separation by thin-layer chromatography revealed that sarcolemma contains mainly cholesterol and cholesterol esters and a trace of triglycerides (Fig. 2). The phospholipid fraction remains at the point of origin.

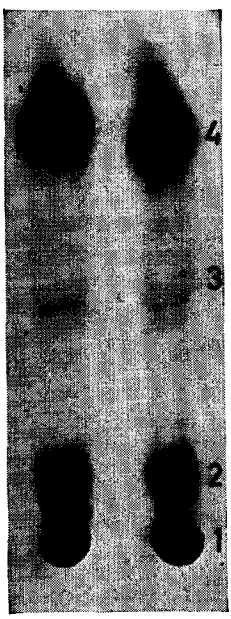

Fig. 2. Separation of neutral lipids by thin-layer chromatography. The phospholipids remain at the origin (1). The other spots correspond to cholesterol (2), triglycerides (3) and cholesterol esters (4).

Separation and determination of phospholipids

The chromatograms run with chloroform–methanol–acetic acid–water(25:15:4:2, by vol.) show the various phospholipids (Fig. 3). The comparison of chromatograms of samples with those of standards revealed that sarcolemma contains lysolecithin, sphingomyelin, lecithin and phosphatidylethanolamine (Fig. 3). A spot remains at the point of origin and could not be identified.

The quantitative determination of phospholipids by analyses of the inorganic phosphate in digested samples gave the results summarized in Table II. The left column of this table accounts for the percentage of phosphate content of each phos-

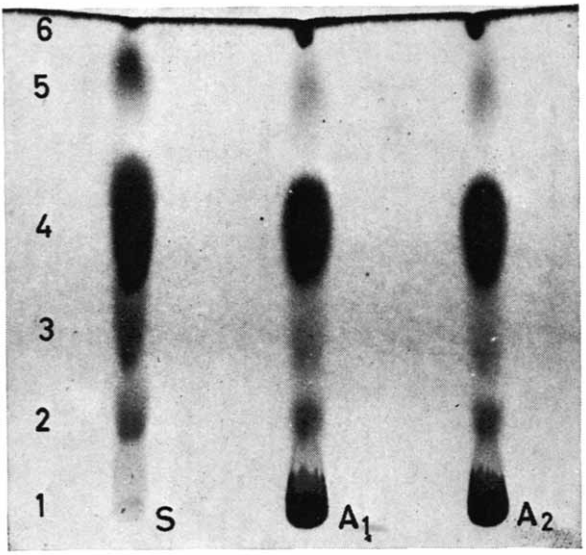

Fig. 3. Separation of phospholipids into classes. The left pattern (S) corresponds to the separation of a standard mixture where the separated spots are lysolecithin (2), sphingomyelin (3), lecithin (4), phosphatidylethanolamine (5) and phosphatidylserine (6). The patterns A_1 and A_2 refer to the separation of samples. Spot 1 was not identified.

TABLE II

PHOSPHOLIPID COMPOSITION OF THE SARCOLEMMA ISOLATED FROM RABBIT SKELETAL MUSCLE

Phospholipid	*% total phosphate*	*% of total phospholipid by comparison with standards*
Lysolecithin	11.64±1.83 (4)	9.45±2.23 (4)
Sphingomyelin	8.37±2.70 (4)	23.26±2.16 (4)
Lecithin	66.82±3.70 (4)	43.07±7.45 (4)
Phosphatidylethanolamine	8.69±1.22 (4)	24.25±2.56 (4)
Phosphatidylserine	0.46±0.05 (4)	Trace
Origin	5.38±1.55 (4)	

pholipid. In the right column is presented the relative amount of each phospholipid by comparison of the phosphate content of samples with the phosphate content of standards.

Separation of proteins and determination of their molecular weights

Using sodium dodecyl sulfate–polycrylamide gel electrophoresis we were able to separate about twelve main protein bands with molecular weights ranging from 12000 to 200000 (Fig. 4). At the top of the gels a stationary band is observed which probably corresponds to the collagen fraction of sarcolemma whose molecular weight is very high[34]. The following band of low mobility has approximately the same mobility of myosin and probably represents myosin contamination in our preparations.

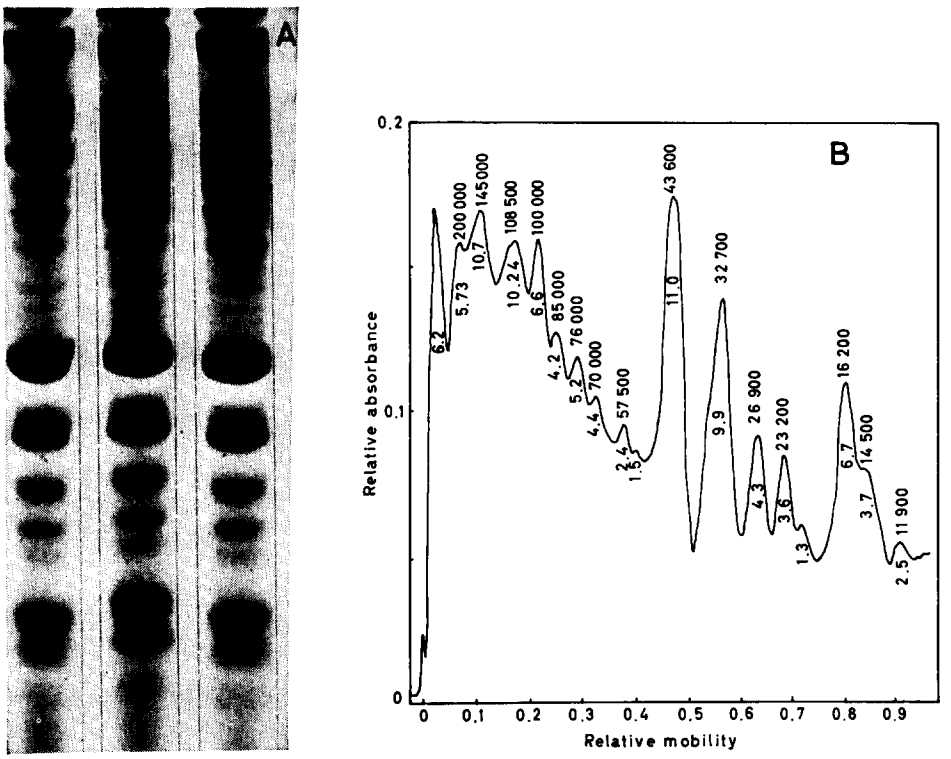

Fig. 4. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of sarcolemmal proteins. (A) Polyacrylamide gels stained with Coomassie blue. (B) Continuous record of the absorbance of the gels. We refer the molecular weights above the peaks and the relative amount (percentage) of each band under the peaks.

We attempted to characterize the collagen band (or bands). For this purpose we digested suspensions of fragmented sarcolemma with collagenase (Sigma) using the method of Kono *et al.*[17]. Digested samples were analysed electrophoretically and we could not detect any protein band. Furthermore, we also digested other sub-

cellular fractions such as mitochondria and myosin and, in all cases, digestion with collagenase caused disappearance of all the bands detected in controls. Apparently the collagenase used contains proteases other than collagenase which are responsible for the degradation of the proteins, or the sarcolemmal proteins are all collagenase labile.

The integration of the scanned records of the gels gives us information about the relative amount of each protein, which is summarized in Fig. 4.

DISCUSSION

The chemical composition for the sarcolemmal membranes as determined by us is in close agreement with the results reported by other authors for sarcolemma isolated from animals other than rabbits[17,36–38]. Recently Fiehn and Peter[38] have reported the lipid composition of the sarcolemma isolated from rat skeletal muscle and their results are similar to those obtained by us except for sphingomyelin and phosphatidylserine. Those authors report values of 14.4 and 17.6% for sphingomyelin and phosphatidylserine, respectively, while in our studies we detected 23% of sphingomyelin and only a trace amount of phosphatidylserine. Furthermore, we found the presence of lysolecithin in all the preparations and its amount does not vary appreciably from preparation to preparation ($11.6 \pm 1.8\%$). These differences may result from the methods applied and also from the biological material itself which was obtained from rats in the work of Fiehn and Peter[38] and from rabbits in our own work.

Acrylamide gel electrophoresis of the sarcolemmal proteins revealed surprisingly that collagen probably represents only a small fraction of the total protein. The ultrastructure of isolated sarcolemma has been described by several authors[16–19] who suggest that collagen represents a major component of sarcolemma. However, other authors[36] working with isolated sarcolemma from bullfrog muscles observed that isolated membranes have a small portion of collagen and these authors believe that the major portion of collagen is removed by the washing procedure with hypertonic KCl during the preparation of membranes. Our material was also washed with 0.6 M KCl and this may be the reason for the disappearance of collagen. Furthermore, the conclusion of Kono *et al.*[17] that collagen is a major component of sarcolemma was based on the digestion of sarcolemma by collagenase which caused collagenase-labile protein to disappear. As suggested by our results obtained with collagenase, the enzyme preparation may not be specific for collagen. Our results reveal that the collagenase we used (obtained from Sigma) digests almost all of the sarcolemmal protein, since after digestion we could not detect any protein in the polyacrylamide gels. We have also demonstrated that collagenase digests myosin and mitochondrial proteins.

On the other hand, the structural studies we made with the electron microscope reveal that the major structural component is the basement membrane (Fig. 1). If we assume that collagen is not a major component of isolated sarcolemma it seems reasonable to think that this membrane is formed by substances other than collagen.

The nucleic acid fraction (3–5%) found in sarcolemma may be derived from contamination by adhering nuclei which were not removed during the isolation of membranes.

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