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MUSCLE SURFACE MEMBRANES

PREPARATIVE METHODS AFFECT APPARENT CHEMICAL PROPERTIES AND NEUROTOXIN BINDING

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Summary

Considerable disagreement exists between results reported by various authors for lipid composition and enzyme activity in purified muscle membrane fractions presumed to be sarcolemma, although an explanation for these discrepancies has not been presented. We have prepared muscle light surface membrane fractions of comparable density (1.050—1.120) by a low-salt sucrose method and by an LiBr-KCl extraction procedure and compared them for density profile, total lipid and cholesterol content, protein composition and ATPase activity. In addition, sodium channels characteristic of excitable membranes have been quantitated in each preparation using [^{3}H]saxitoxin binding assays, and the density of acetylcholine receptors determined in fractions from control and denervated muscle using α-[^{125}I]bungarotoxin. Although both fractions contain predominantly surface membrane, the LiBr fraction consistently shows the higher specific activity of *p*-nitrophenylphosphatase, higher free cholesterol content, and higher density of sodium channels and acetylcholine receptors. The density distribution of sodium channels appears uniform throughout both fractions. Quantitative differences were seen between sodium dodecyl sulfate-polyacrylamide gel electrophoresis patterns of membrane proteins from the two preparations although most bands are represented in both. A majority of the low-salt sucrose light membrane proteins were accessible in varying degrees to labelling with diazotized diiodosulfanylic acid in intact muscle.

These results suggest that light surface membrane fractions may be mixtures of sarcolemma and T-tubular membranes. Using our preparative methods, the LiBr fraction may contain predominantly sarcolemma while low-salt sucrose light membranes may be enriched in T-tubular elements.

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Introduction

Isolation of muscle surface membrane requires its separation from the normally insoluble contractile elements of the cell as well as from nuclei, mitochondria and membranes of the sarcoplasmic reticulum. A number of isolation procedures have been reported in recent years for mammalian skeletal muscle which rely on prolonged extraction with 0.4 M LiBr followed by 0.6 M KCl for solubilization of contractile proteins as an integral component of the membrane purification procedure [1—3]. Several other methods attempting to avoid this rigorous salt extraction have been reported from this and other laboratories [4—6]; in our initial procedure differential centrifugation on low-salt sucrose gradients followed by equilibrium centrifugation on a continuous gradient was used [6]. In others, an incubation at 37°C was introduced prior to actomyosin extraction [5].

In most preparations in which membrane densities ultimately have been characterized, presumed surface membrane markers appear at highest specific activity in those fractions banding between 20 and 30% sucrose independent of the method used to separate membranes from contractile protein. Reported values for these markers, however, vary over a wide range among these methods. Since in most cases the membrane product is presumed to be sarcolemma, this wide variability in normal values is disturbing.

Within the system of muscle membranes, surface membrane must first be differentiated from elements of the sarcoplasmic reticulum. Once surface membrane has been tentatively identified, a further difficulty arises in distinguishing between true sarcolemma and membranes arising from the T-tubular system. Both membranes are continuous [7]; both are accessible to surface-specific probes in the extracellular space [8]; both presumably contain similar unique functional molecules such as sodium channels [9]. Further, a recent study has reported the identification and isolation of T-tubular membrane fragments which themselves band between 22 and 26% on a sucrose density gradient, emphasizing the potential difficulty in differentiating between sarcolemmal and T-tubular elements in the various preparations referred to above [10].

In the present study a modification of our low-salt sucrose procedure [6] is described for the rapid preparation of light muscle surface membranes, and a method for the preparation of membranes of similar density using a modified LiBr-KCl procedure is presented. These light membrane fractions are then analyzed and compared for the presence of a number of physiologic identifiers of surface membrane including [^{3}H]saxitoxin binding sites and acetylcholine receptors, as well as for ($Na^+ + K^+$)-ATPase activity, cholesterol content, and protein composition. On the basis of comparisons of these and other biochemical parameters, and utilizing data from recently reported work, it appears that the light membrane fraction isolated by these procedures represents surface membrane containing a variable mixture of T-tubular and sarcolemmal elements. Membranes isolated using the low-salt sucrose procedure appear to be relatively enriched in T-tubular components while membranes of the same density isolated using LiBr and KCl extraction appear enriched in sarcolemmal elements. Since both approaches to the isolation of muscle surface membrane probably yield a mixture of T-system and sarcolemmal elements and both

parallel to varying degrees previously reported preparative methods, interpretation and comparison of sarcolemmal analyses reported in the literature must be carried out with caution.

Methods

Preparation of light surface membrane by the low-salt sucrose procedure. 25—40 g fresh rat muscle was minced on ice with a razor blade, transferred to 10 vols. cold buffer A (50 mM Ca^{2+}/150 mM sucrose/5 mM Tris pH 7.3), and homogenized for 7 s in a Waring blendor. The homogenate was filtered through cheesecloth to remove connective tissue and the residue rehomogenized in buffer A and refiltered if necessary. The filtrate was centrifuged for 5 min at 2000 $\times g$ and the resultant pellets resuspended in cold buffer B (30% (w/w) sucrose/5 mM Ca^{2+}/5 mM Tris, pH 7.3). The suspension was homogenized with 10 strokes of a Teflon-pestle homogenizer and then centrifuged for 10 min at 20 000 $\times g$. The supernatant was collected on ice, the pellets resuspended in buffer B and rehomogenized. This cycle is repeated 3 times and all supernatants combined. Dilution to 10% sucrose and pelleting of all material at this point yields a crude low-salt sucrose membrane fraction which could be fractionated on a continuous gradient. For routine preparation of light membranes, the combined supernatant was instead diluted to 28.5% sucrose by refractometer and overlayered with 15% sucrose/5 mM Tris, pH 7.3, to form a discontinuous gradient; the gradients were centrifuged at 100 000 $\times g$ for 90 min. Material banding at the 15/28% interface was collected and washed; this material was designated LSS light membranes.

Preparation of light surface membranes by the LiBr-KCl method. 30 g fresh muscle was minced, homogenized in 0.25 M sucrose/0.2 mM EDTA/0.1 M Tris (pH 7.6) in a Waring blendor for 7 s, centrifuged at 1000 $\times g$ for 10 min, and brought to a final volume of 5 times the original weight with a LiBr solution such that the final concentration was 0.4 M LiBr, 0.01 M Tris, (pH 8.5). This suspension was stirred gently in the cold for 12—14 h. Extracted membranes were then harvested by the method of Festoff and Engel [3] and a crude membrane pellet was obtained. For isolation of LiBr-light membranes, this fraction was washed with deionized water, resuspended in 28.5% sucrose/Tris (pH 7.3), overlayered with 15% sucrose and centrifuged at 100 000 $\times g$ for 90 min. The resultant interfacial material which was collected and pelleted was designated LiBr-light membranes.

[^{3}H]Saxitoxin binding. Saxitoxin was a generous gift of Dr. E.J. Schantz of the University of Wisconsin. [^{3}H]Saxitoxin was prepared by exchange tritiation by New England Nuclear Co. after the method of Ritchie et al. [11]. Crude [^{3}H]saxitonin was purified as necessary on columns of Sephadex G-15 followed by analysis on BioRex 70 cation exchange resin. Bioassay was carried out on desheathed frog sciatic nerve in a sucrose gap recording system [12]. Specific activities for resultant [^{3}H]saxitoxin ranged between 20 and 25 Ci/mmol and radiopurities between 60 and 75%.

Binding assays on membrane fractions were carried out by incubation of [^{3}H]saxitoxin at various concentrations with purified membrane in the presence or absence of excess cold tetrodotoxin ($1 \cdot 10^{-6}$ M). Bound toxin was

separated from unbound by rapid filtration on glass fiber filters as described by us elsewhere and bound activity quantitated by liquid scintillation counting [13]. Concentration-dependent binding curves were then constructed for both non-specific and specific binding. Determinations on homogenates were done using rapid centrifugation to separate particulate material from the incubation mixture; pellets were subsequently solubilized in 5% SDS and both supernatant and pellets were counted. Again, specific binding was determined by competition with excess cold tetrodotoxin. In either case, concentration of binding sites was expressed in terms of pmol [^{3}H]saxitoxin bound specifically per mg protein.

Purified membranes were solubilized by suspension at 1 mg/ml final concentration in 1% lubrol-PX or 1% Brig 96/98 (1 : 1 mixture), 50 mM K_2HPO_4 buffer, pH 7.6 (Weigele, J. and Barchi, R., in preparation). The suspension was gently homogenized at 0°C in a Teflon-glass homogenizer. [^{3}H]Saxitoxin binding to the solubilized receptor was determined by addition of $7.5 \cdot 10^{-9}$ M [^{3}H]saxitoxin in the presence or absence of 10^{-6} M unlabeled, tetrodotoxin. Toxin bound to the solubilized channel was separated from free toxin by centrifugation through short (4 cm) columns of Sephadex G-25 at 0°C after the method of Lefkowitz et al. [14].

Acetylcholine receptor. Density of acetylcholine receptor was determined in isolated membrane fractions after the method of Andrew et al. [15]. α-[^{125}I]-Bungarotoxin was prepared by using chloramine-T and purified by subsequent chromatography on Sephadex G-25 and CM-25 [16]. Binding was carried out on membrane fractions solubilized in Triton X-100 and bound toxin separated from free by chromatography on detergent-equilibrated G-100 columns. In all cases, specificity of binding was monitored by parallel incubations containing excess *d*-tubocurarine. Binding site density was expressed as pmol α-bungarotoxin per mg membrane protein.

Membrane p-nitrophenylphosphatase. K^+-stimulated acyl phosphatase was assayed spectrophotometrically by continuous measurement of the conversion of *p*-nitrophenylphosphate to *p*-nitrophenol. Reaction mixtures contain 3 mM *p*-nitrophenylphosphate, 3 mM $MgCl_2$, 0.1 mM EGTA, 0.005% deoxycholate, 50 mM Tris (pH 7.8), membrane protein (25—100 μg), and either 0 or 20 mM KCl; water was added to a final volume of 1 ml. The reaction was initiated by addition of *p*-nitrophenylphosphate after pre-equilibration of all components at 37°C in cuvettes with and without K^+ blanked against a cuvette containing all components except membrane protein. Activity was expressed as initial rate of hydrolysis of *p*-nitrophenylphosphate specifically stimulated by 20 mM K^+ in μmol *p*-nitrophenylphosphate/mg protein $\cdot$ h^{-1}.

(SDS)-Polyacrylamide gel electrophoresis. Membrane proteins were solubilized by heating at 100°C for 3 min in 1% SDS and 0.1% β-mercaptoethanol and separated on 10% acrylamide gels containing 0.2% SDS after the method of Laemmli [17]. Lactoperoxidase iodination of intact muscle was carried out as previously described [6]. Diazotized di[^{125}I]iodosulfanylic acid was prepared as recommended by the suppliers; labelling with this agent was carried out at 10°C in glucose-Ringer's for 60 min using intact EDL which were subsequently washed and processed as for lactoperoxidase iodination. Autoradiograms were prepared from dried SDS-polyacrylamide gels.

Free cholesterol determinations. Total membrane lipids were extracted by the method of Bligh and Dyer [18]; known amounts of cholestanol were added to the membrane suspension prior to lipid extraction as a internal standard. Sterols were analysed by gas-liquid chromatography on a 6 foot column of OV-17 on gas-chrom Q support at 250°C. Peaks were identified by co-chromatography with standards, and free cholesterol content was calculated relative to the internal standard.

Results

A number of the procedures reported for the isolation of muscle 'sarcolemma' or muscle 'surface membranes' are summarized in Table I with banding density of the final membrane preparation, $(Na^+ + K^+)$-ATPase activity, and cholesterol content where available. A wide range of values is seen in both ATPase and cholesterol content for membranes of apparently similar density and identified in most cases by the authors as 'sarcolemma'. In general, methods using extraction with LiBr and KCl [1—3] yield membranes with high ATPase activity and high cholesterol content while differential centrifugation methods with [5] or without [4,6] incubation at 37°C yield membranes of similar density with lower ATPase activity and cholesterol content.

In order to pursue these apparent inconsistencies and to further characterize the membrane isolated by our previously reported procedure [6], we compared presumptive muscle surface membrane prepared using a modification of our low-salt sucrose method (see Methods) with that prepared by the LiBr-KCl extraction technique. Crude membrane fractions isolated from rat skeletal muscle using the low-salt sucrose procedure equilibrate into three distinct fractions on a continuous 15—45% sucrose density gradient (Fig. 1A). In an earlier study we demonstrated that the fraction banding between 19 and 29% sucrose could be labeled to highest activity with surface-specific probes and contained the highest specific activities of enzymes usually associated with the plasma membrane [6]. When crude muscle membranes are isolated using a modification of the LiBr extraction procedure of Festoff and Engel [3] and centrifuged to equilibrium on an identical gradient, a similar profile is obtained (Fig. 1B), as was previously reported by those authors. We confirm their observation that markers for the surface membrane in this preparation also appear in highest specific activity in those membranes banding at sucrose densities less than 30%.

Since the membranes of interest in both preparations corresponded to the fraction banding between 19 and 29% sucrose, modified isolation procedures were developed (see Methods) for the rapid preparation of a light membrane fraction from either low-salt sucrose or LiBr-KCl crude membranes without the necessity for equilibrium sedimentation on a continuous gradient. When these fractions were collected, washed, and subsequently spun to equilibrium on 14—45% continuous sucrose gradients, each banded as a discrete peak at the same position on the gradient as the lightest membrane fraction from their respective original preparations (Fig. 1, C and D). These fractions, termed LSS light and LiBr light membranes, were used exclusively for subsequent comparative studies.

Both light membrane fractions were re-run on shallow (15—30%) sucrose

TABLE I

SUMMARY OF VALUES FOR MEMBRANE CHOLESTEROL AND $(Na^+ + K^+)$-ATPase FROM THIS STUDY AND FROM REPORTS IN THE LITERATURE FOR LIGHT MUSCLE MEMBRANES CONSIDERED TO BE PURIFIED SARCOLEMMA OR SURFACE MEMBRANE

Study	Tissue	Isolation procedure	Banding density (% sucrose)	ATPase activity (μmol/mg protein per h)	Cholesterol (μg/mg protein)
Fiehn et al. [19]	Rat skeletal	Incubation at 37°C EDTA extraction	—	2.7	156
DeKretser and Livett [20]	Mouse skeletal	Incubation at 37°C EDTA extraction	—	3.3	3.5
Kidwai et al. [4]	Rat skeletal	One-step continuous sucrose gradient	8—30	15.0	69.6
Schapira et al. [2]	Rat skeletal	LiBr, KCl extraction	36/41 interface	11.1	133
Andrew and Appel [1]	Rat skeletal	LiBr, KCl extraction continuous gradient	Fraction I, 20—29 Fraction II, 20—29	20.4 5.2	— —
Festoff and Engel [3]	Rat skeletal	LiBr, KCl discontinuous gradient	≈24	48.7	—
Barchi et al.	Rat skeletal	LiBr, KCl discontinuous gradient	22—26	45.2	188
Barchi et al.	Rat skeletal	Low-salt sucrose discontinuous gradient	22—26	4.7	48
Lau et al. [10]	Rat skeletal (T-tubule)	French pressure cell sucrose gradient	21—26	6.2	—

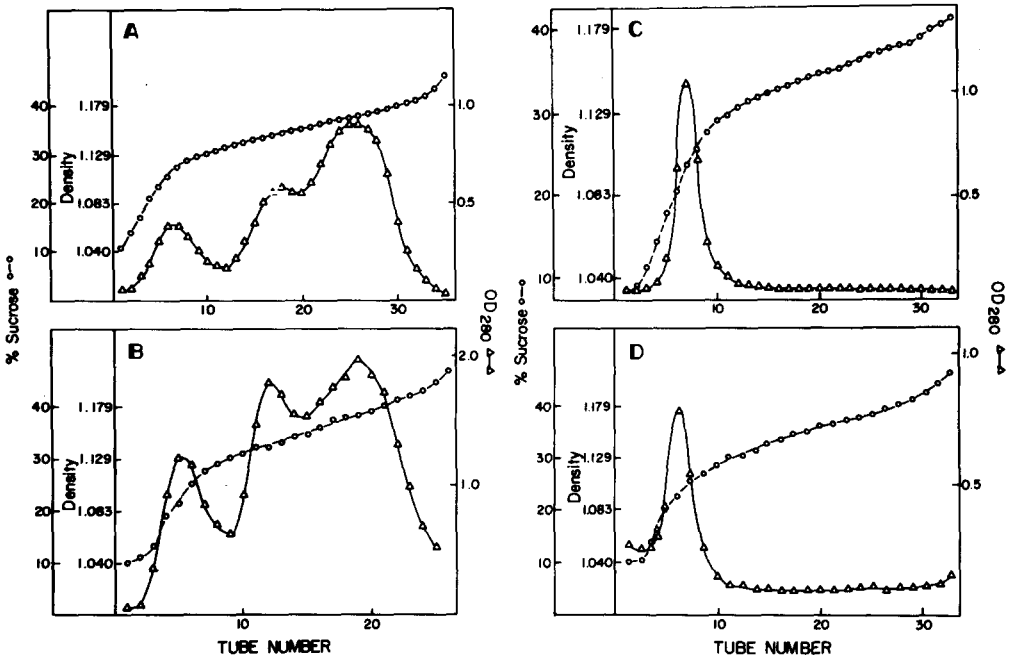

Fig. 1. Distribution of protein on continuous sucrose gradients following the various membrane preparative procedures. A. Crude membrane fraction prepared by low-salt sucrose method. B. Crude membrane fraction prepared by LiBr-KCl extraction. C. Light membranes from modified low-salt sucrose procedure. D. Light membrane from modified LiBr-KCl procedures. All gradients were 15—45% w/w sucrose and 5 mM Tris, pH 7.3. Centrifugation, 15 h at 100 000 × *g*.

gradients for determination of average peak densities. Low-salt sucrose membranes exhibited a major peak at 24.1 ± 0.9% sucrose (8 preparations) and a small shoulder (always less than 10% of total protein) at 17.9 ± 0.7%. Similar patterns were obtained with LiBr light membranes with a principal peak at 23.5 ± 1.2% sucrose and a variable minor peak at 18.2 ± 0.9%.

Muscle used for preparations of membranes. In the studies reported below, unselected muscle from the rat hind limb was used as the starting material for membrane preparation unless otherwise indicated. Most of these limb muscles demonstrate fast twitch kinetics and contain a mixture of fast and slow fibers with fast fibers predominating. Several (e.g. extensor digitorum longus) are composed almost entirely of fast twitch fibers [21]. The soleus, a minor component by weight, contains predominantly slow twitch fibers [21]. Several membrane preparations were carried out with both low-salt sucrose and LiBr techniques using selected fast (extensor digitorum longus) or slow (soleus) muscle as starting material. Membranes prepared from extensor digitorum longus behaved similarly on sucrose gradients to those prepared from whole leg muscle and were also comparable with respect to the studies presented below, reflecting the dominant fast characteristics of the limb muscles in question. A similar observation was made in our earlier report on the low-salt sucrose membrane preparation from rat muscle [6]. In agreement with the results of others, membranes prepared from soleus differed from those prepared from extensor digitorum longus or whole limb muscle using either isolation technique in

regard to sucrose density profile and polyacrylamide gel protein patterns [22]. Since most of the isolation procedures compared in Table I use either unselected muscle [2,4,19,20] or muscle of mixed fiber type [1,3] as starting material, and since we found no significant differences using either low-salt sucrose or LiBr isolation methods between unselected hind limb muscles and selected extensor digitorum longus, the former was used as starting material in most experiments. The differences between pure slow and pure fast muscle membrane, although of considerable interest, will not be discussed further here.

p-Nitrophenylphosphatase activity. Activity of the membrane ($Na^+ + K^+ + Mg^{2+}$)-ATPase was estimated in 9 preparations of LSS-light membranes and 19 preparations of light membranes obtained with the LiBr-KCl method. K^+-Activatable *p*-nitrophenylphosphatase activity was used as a measure of this membrane enzyme [23,24]. Specific activities for K^+-activated *p*-nitrophenylphosphatase averaged 4.7 μmol P_i/mg protein $\cdot$ h^{-1} for LSS-light membranes and 45.2 μmol P_i/mg protein $\cdot$ h^{-1} for LiBr-light membranes. Comparison of these values with others reported in the literature for various isolation techniques is summarized in Table I.

We subsequently determined the distribution of *p*-nitrophenylphosphatase activity within the light membrane fraction for both isolation procedures following separation on a shallow sucrose gradient. The protein and enzyme distribution for salt extracted membranes is shown in Fig. 2A. *p*-Nitrophenylphosphatase activity overlapped the peak of protein concentration, but led it slightly on the gradient. This results in the observation, confirmed in subsequent runs, that the highest specific activity for *p*-nitrophenylphosphatase is found in membranes on the leading edge of the protein peak and that the

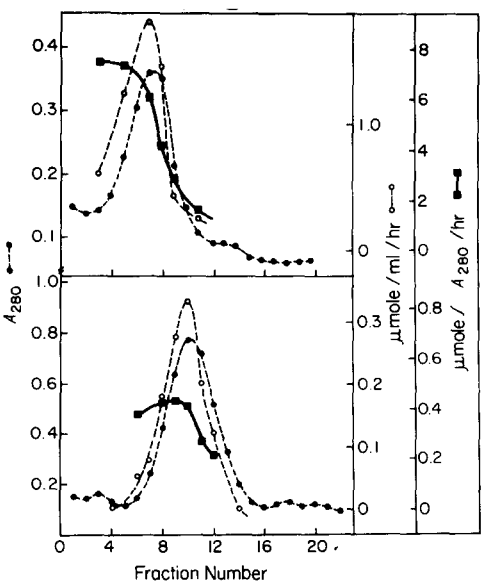

Fig. 2. Protein distribution and *p*-nitrophenylphosphatase activity for light membrane fractions separated on shallow 15—30% sucrose gradients. A, LSS-light membranes; B, LiBr-light membranes. In each case, specific activity of *p*-nitrophenylphosphate is slightly higher at the leading edge of the protein peak.

specific activity decreases slightly as the density increases. A parallel experiment using LSS-light membranes is shown in Fig. 2B; again the distribution of membrane protein is similar to that seen for the LiBr-extracted material. Sequential *p*-nitrophenylphosphatase assays indicated that the peak of enzyme activity here also led slightly the peak of membrane protein concentration on the gradient, yielding highest specific activities in the leading edge of the major membrane protein band. This observation may suggest some slight heterogeneity in both light membrane fractions.

Cholesterol content. Total lipid content and free cholesterol content were determined in a series of 8 preparations of light LSS membrane. In these preparations, total lipid averaged 1.03 mg/mg membrane protein, a value which might be anticipated from the relatively low banding density of this preparation. Similar values were obtained for LiBr membranes. Free cholesterol content was determined in 4 preparations of LSS membranes and 5 preparations of comparable density membranes isolated using LiBr. The average values for these preparations are shown in Table I. It can be seen that cholesterol content in LiBr-light membranes in significantly higher (188 ± 42 μg/mg protein) than in LSS-light membranes (48 ± 16 μg/mg protein). In both cases, however, these values are much higher than those reported for sarcoplasmic reticulum [25,26].

Membrane proteins. Membrane proteins from the light membrane fractions isolated by the low-salt sucrose method and by LiBr extraction were analysed by polyacrylamide gel electrophoresis following solubilization with SDS and β-mercaptoethanol. Banding patterns for the two light membrane fractions are shown in Fig. 3. In the low-salt sucrose procedure, major protein bands were evident at 109 000, 93 000; 75 000; 34 000 and 16 500 daltons. Numerous additional small but reproducible bands were also distinguishable. Membranes isolated by the salt extraction procedure had many distinct bands in common with those of the low-salt sucrose membranes, but their relative intensities differed considerably. Most obvious was the relative reduction in intensity of the 109 000 and 57 000 dalton bands, and the relative augmentation of proteins banding near 75 000 and 51 000 daltons. Traces of high molecular weight protein running in a position comparable to that of native myosin were seen occasionally in both preparations. The gels shown here represent the extreme of variation between preparative methods; often considerably more congruity was seen between the protein patterns.

In a previous report a number of criteria were used to indicate that LSS-light membranes were surface in origin [6]. In order to minimize the possibility of this fraction being composed of predominantly internal membranes with a small fraction of surface membrane carrying labels in high specific activity, a number of experiments were carried out examining the labeling patterns of membrane protein in this fraction in the intact muscle using labels restricted to the extracellular space. Labeling was carried out with ^{125}I and lactoperoxidase as previously reported [6] and with ^{125}I-labeled diazotized diiodosulfanylic acid. After extensively washing the intact muscles, LSS-light membranes were prepared and membrane proteins solubilized and separated by SDS-polyacrylamide gel electrophoresis. Contact autoradiographs were then made of the resultant gels. A typical experiment is shown in Fig. 4 comparing the Coomassie Blue staining pattern for the LSS-light membrane proteins with a densi-

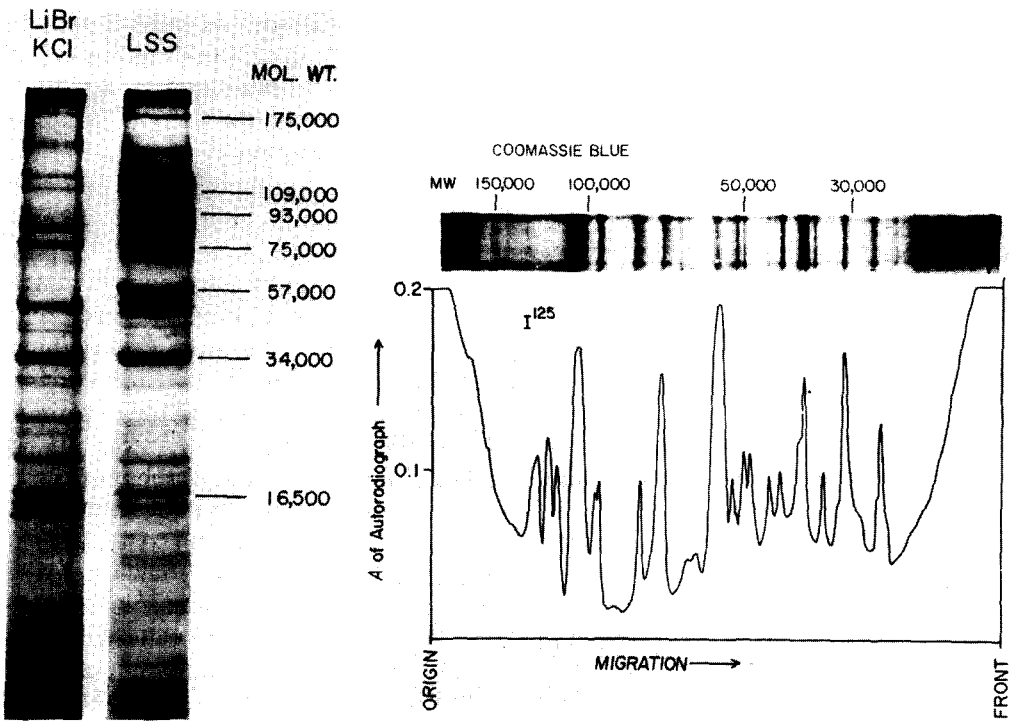

Fig. 3. Membrane proteins separated on SDS-polyacrylamide gel electrophoresis for LSS- and LiBr-light membranes. Most proteins are represented in both preparations but their relative contribution to the total protein content differs markedly. These gels represent the extremes of incongruity observed between the two preparations; more overlap is often seen.

Fig. 4. Labeling of LSS-light membrane proteins in intact muscle using ^{125}I-labeled diazotized diiodosulfanylic acid. Membranes isolated after extensive washing show a normal protein pattern; autoradiography of the resultant gels indicates significant labeling of several bands visible with Coomassie Blue and lesser incorporation into numerous others.

tometer scan of an autoradiogram of the same gel. Several major and numerous minor peaks of radioactivity were seen, all of which correspond to proteins identified by Coomassie Blue staining. Highest incorporation of label was seen in proteins banding at 109 000; 75 000 and 57 000 daltons. Taken in conjunction with the binding data previously reported [6] this supports the contention that the LSS-light fraction contains predominantly membranes which are accessible on the surface of the muscle cell, as opposed to a small fraction of surface membrane co-isolated with a large proportion of contaminating internal membrane.

Saxitoxin binding sites in isolated particular membrane fractions. A characteristic presumed to be specific for the surface membrane of excitable cells is the presence of a voltage-dependent conductance channel for sodium ions which is involved in the generation of the action potential. Physiological evidence suggests that this sodium channel is present in both sarcolemma and T-tubular membrane, although the density of channels is predicted to be 10 times higher in the sarcolemma [9]. Saxitoxin, a small molecule derived from phytoplankton and similar in action to the more familiar tetrodotoxin, binds

with high specificity to the voltage-dependent sodium channel [27,28]. Tritiated saxitoxin has been used in several systems to quantitate the number of sodium channels present using a variety of equilibrium binding assays [11,29].

Determination of [^{3}H]saxitoxin binding to isolated muscle membranes was carried out using a rapid filtration procedure [13]. Measurements were made at increasing [^{3}H]saxitoxin concentrations in the presence or absence of excess unlabeled tetrodotoxin to generate curves indicative of specific and non-specific binding to the membranes. Similar binding curves were constructed for crude muscle homogenates using a centrifugation assay following equilibration of homogenate with various toxin solutions.

Typical saturation curves indicating total and non-specific (excess cold toxin) saxitoxin binding to crude rat muscle homogenate, LSS-light membranes, and LiBr-light membranes are shown in Fig. 5. Scatchard plots of resultant specific toxin binding for each case indicate non-cooperative binding to a single class of sites. The apparent K_d for toxin-channel interaction in homogenate as determined from double reciprocals plots of specific binding (Fig. 5) is $1.5 \cdot 10^{-9}$ M at 0°C. Comparable values of $1.6 \cdot 10^{-9}$ M and $1.5 \cdot 10^{-9}$ M are obtained for LSS- and LiBr-light membranes, respectively. As we described elsewhere for saxitoxin binding to rat synaptosomal sodium channels, values of apparent K_d vary significantly with temperature and ionic strength, becoming larger (lower affinity) at higher temperatures and higher ionic strengths [13].

Total concentration of binding sites in crude homogenate averaged 15 pmol/g wet wt. tissue or 0.12 pmol/mg protein in the homogenate. Values for the

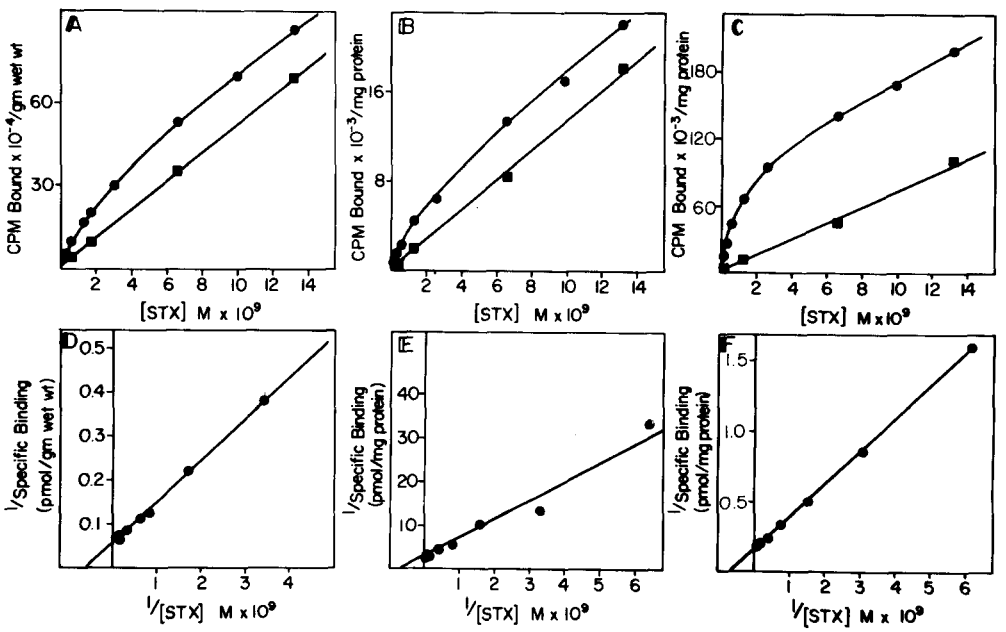

Fig. 5. Total and nonspecific binding of [^{3}H]saxitoxin to (A) rat muscle homogenate, (B) LSS-light membranes and (C) LiBr-light membranes. Double reciprocal plots of specific binding in each case (D—F) indicate an apparent K_d for binding of between $1.5 \cdot 10^{-9}$ and $1.8 \cdot 10^{-9}$ M at 0° C. STX, saxitoxin.

LSS-light fraction averaged 0.46 pmol/mg membrane protein while those for LiBr-light membranes averaged 6.8 pmol/mg protein. This represents an apparent purification of 4-fold in the former case and 56-fold in the latter. Values of saxitoxin binding to isolated muscle membranes are compared with similar values reported for other isolated membrane preparations in Table II.

The distribution of [^{3}H]saxitoxin binding within the LiBr- and LSS-light membrane fractions was further examined as a function of density following equilibrium centrifugation of each fraction on shallow continuous sucrose gradients. As illustrated in Fig. 6 for LiBr-light membranes, the peak of toxin binding paralleled the membrane protein distribution. Specific activity for toxin binding appeared uniform and did not suggest the existence of marked heterogeneity within the fraction.

Saxitoxin binding in solubilized membrane fractions. An important consideration regarding the observed differences between LSS and LiBr membranes is the potential contribution of vesicle sidedness to the accessability of the enzyme molecules and receptor proteins being assayed. Thus, the formation of predominantly inside-out vesicles in the low-salt sucrose procedure could yield anomalously low values of [^{3}H]saxitoxin binding since this highly charged molecule would not be expected to cross the vesicle membrane and since binding is known to occur only at the outer surface of the native membrane [30]. This question was addressed by solubilizing membrane vesicles in detergent in a manner which destroys membrane integrity but preserves saxitoxin binding capacity and again comparing binding between LSS and LiBr membranes. The characteristics of the solubilized saxotoxin binding component of the muscle membrane sodium channel will be presented in detail in a subsequent report (Weigele, J. and Barchi, R., in preparation).

Membranes from both preparations usually retained 35—50% of their saxitoxin binding capacity after solubilization in 1% Lubrol PX or 1% Brij 96/98

TABLE II

SODIUM CHANNEL DENSITY IN ISOLATED EXCITABLE MEMBRANES AS DETERMINED BY [^{3}H]SAXITOXIN OR [^{3}H]TETRODOTOXIN BINDING

Values from isolated muscle surface membrane as determined in this study are compared with representative values for other membrane systems reported in the literature.

Study	Preparation	Ligand	Site concentration (pmol/mg protein)
This study	Rat muscle LiBr-light membranes	[^{3}H] Saxitoxin	6—7
This study	Rat muscle LSS-light membranes	[^{3}H] Saxitoxin	0.35—0.50
Reed and Raftery [42]	Electroplax isolated surface membrane	[^{3}H] Tetrodotoxin	2—3
Balerna et al. [43]	Spider crab isolated axolemma	[^{3}H] Tetrodotoxin	6—7.5
Balerna et al. [43]	Lobster isolated axolemma	[^{3}H] Tetrodotoxin	11—12.5
Chacko et al. [44]	Garfish oldfactory nerve isolated axolemma	[^{3}H] Tetrodotoxin	3.7

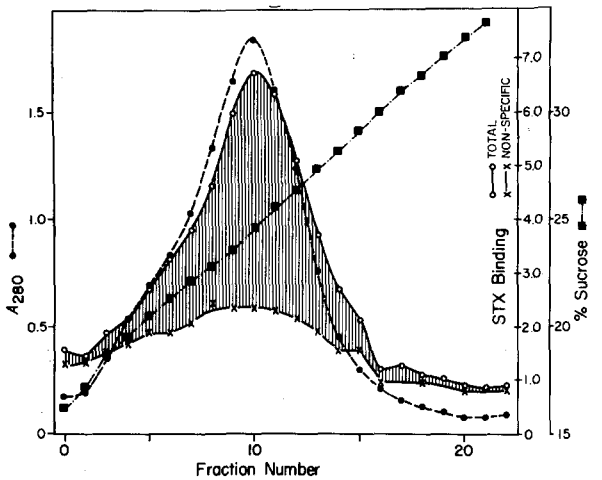

Fig. 6. Distribution of [^{3}H]saxitoxin binding sites within the LiBr-light membrane fraction following separation on a shallow (15—30%) sucrose gradient. Non-specific and total binding determinations were carried out on sequential fractions; the difference between these values (shaded area) represents specific saxitoxin (STX) binding. Toxin binding parallels protein distribution and the calculated density of sodium channels per mg protein remains fairly uniform throughout the peak.

50 mM K_2HPO_4, pH 7.6. Determinations of specific binding with saturating concentrations of saxitoxin in the presence or absence of excess unlabeled tetrodotoxin demonstrated binding site densities of 0.142 pmol/mg protein for two preparations of LSS membranes and 3.32 pmol/mg protein in three preparations of LiBr membranes. The ratio of binding site densities between the two preparations was not significantly altered by solubilization indicating that the majority of saxitoxin binding sites must have been exposed on the external surface of the original vesicles. These findings indicate that the presence of inside-out vesicles does not contribute significantly to the differences observed between LSS and LiBr membranes.

Acetylcholine receptor density. It is widely accepted that the acetylcholine receptor in muscle is localized on the sarcolemma; denervation has been shown repeatedly to produce a marked rise in the density of receptors in non-junctional surface membrane under physiologic conditions [31,32]. α-Bungarotoxin was used in a series of binding studies to quantify the density of acetylcholine receptors in the light surface membranes prepared by the low-salt sucrose procedure under control conditions and following denervation. For these binding studies, only the gastrocnemeus muscle and muscles from the anterior tibial compartment (e.g. those muscles innervated by the sciatic nerve exclusive of the soleus) were used for membrane preparation. In each animal the sciatic nerve in one leg was transected, the other leg served as a source of control muscle.

Values for α-bungarotoxin binding to LiBr membrane fractions under comparable conditions have already been reported by others [15]. Binding studies were carried out following solubilization of isolated membranes in Triton X-100 (see Methods). In a total of 5 separate low-salt sucrose preparations of surface membrane from control animals, an average of 0.35 pmol α-bungaro-

TABLE III

BINDING OF α-[^{125}I]BUNGAROTOXIN MEASURED IN LSS-LIGHT MEMBRANES BEFORE AND 10 DAYS AFTER DENERVATION AS COMPARED WITH SIMILARLY DETERMINED VALUES REPORTED FOR A LiBr-KCl LIGHT MEMBRANE PREPARATION

Preparation		α-[^{125}I]bungarotoxin bound (pmol/mg protein)	
		Normal	Denervated
LSS-membranes		0.35	3.19
LiBr-KCl			
Andrew and Appel [1]	Fraction I	1.04	16.87
	Fraction II	0.27	5.30

toxin binding sites per mg membrane protein was found (Table III). Following 8 days of denervation the density of α-bungarotoxin binding sites increased approximately 10-fold, averaging 3.10 pmol binding sites/mg protein in 6 preparations. The range of binding site densities following denervation was greater than the range noted in control muscle, suggesting the possibility of sub-total denervation in some of the muscles isolated.

Since all determinations of α-bungarotoxin binding were carried out on solubilized membrane, differing percentages of inside-out vesicles in the low-salt sucrose and LiBr preparations could not be contributing to the observed differences in the density of toxin binding sites. These values are compared in Table III with similar acetylcholine receptor densities calculated by Andrew et al. 1[5] in normal and denervated muscle light membrane (20—29% sucrose) isolated by their LiBr-KCl technique. It can be appreciated that the values obtained for light membranes isolated by the low-salt sucrose technique are intermediate between those determined by the above authors for membranes isolated from their microsomal fraction and similar membranes isolated from their heavier nuclear fraction.

Discussion

Muscle membranes isolated by the low-salt sucrose procedure which band at densities between 1.058 and 1.100 appear to be predominantly surface in origin. This can be inferred from data previously presented using lactoperoxidase-catalyzed iodination, binding of ^{125}I-labeled wheat germ agglutinin, and electron histochemical localization of concanavalin A binding as specific markers for surface membrane [6] and is supported by the demonstration in the present study that a majority of the proteins present in this fraction are labelable to varying degrees by probes confined to the extracellular space. There are, however, significant differences in concentration of other markers thought to be specific for surface membrane between this preparation and preparations of membrane of similar density isolated with LiBr-KCl salt extraction. Similar variations are found for these values elsewhere in the literature. How can these differences be reconciled?

Muscle 'surface membrane', when defined in terms of accessibility to markers restricted to the extracellular space, and the presence of ($Na^+ + K^+ +$

Mg^{2+})-ATPase and toxin-binding sodium channels, must be considered to include both the sarcolemma and the T-tubular system. Electron microscopic data indicate continuity of the T-tubular membrane with the sarcolemma and demonstrate the accessibility of the space enclosed by the T-system to large macromolecules in the extracellular space [7,8]. Physiological data document the active propagation of action potentials through the T-tubular system inferring the presence of voltage-dependent sodium channels [9]. Estimates of the density of sodium channels per unit area of T-tubular membrane based on physiologic data range from 10% to 25% of the density of these channels in the sarcolemma, but these values are still the subject of debate [33]. Since the T-tubules and sarcolemma are continuous and both represent surface membrane, it might be anticipated that their lipid/protein ratio and, hence, their banding density on a sucrose gradient would be similar if not identical. Thus, one possible interpretation of our observed differences in marker activities in the LSS membranes and LiBr membranes, as well as the variability of similar values reported in the literature for various preparative techniques, could be a variable ratio of T-tubular to sarcolemmal elements present in the final membrane fraction.

Most LiBr isolation procedures concentrate membranes following actomyosin extraction by attempting to spin down large sheets of sarcolemma still associated with basement membrane complex at low centrifugal forces prior to further purification on sucrose gradients. In the low-salt sucrose procedure membrane fragments are broken up early by vigorous homogenization and separated from denser intact actomyosin filaments by their ability to remain suspended in a 30% sucrose solution at moderate centrifugal forces. Thus, in the LiBr procedure, elements of the T-system, if sheared from the sarcolemma at their tenuous point of contact, would remain in suspension at low centrifugal forces and be separated from the resultant final surface membrane fraction. In the low-salt sucrose, both T-tubular and sarcolemmal membrane would be expected to be broken into smaller fragments with much of the sarcolemma already separated from basement membrane. If both are of low density, they would be co-extracted in 30% sucrose and copurified. Since the T-tubular systems accounts for approximately 8 times as much membrane area per muscle cell as does sarcolemma [28], it might be predicted that the LSS-light membrane fraction would contain significant, if not predominant, amounts of T-tubular system while the LiBr procedure could contain a larger fraction of true sarcolemma.

Direct evidence that T-tubular membranes may band between 22 and 25% sucrose following isolation has recently been presented by Lau et al. using entrapment of ouabain as a marker for vesicles formed from the T-system in the region of the triads [10]. Morphological evidence is presented to support these observations, and electronmicrographs of LSS-light membranes from the present study show frequent profiles similar to those identified by Lau et al. [10] as T-tubular elements.

The specific activities for *p*-nitrophenylphosphatase in the LSS-light membranes is comparable to that obtained for 'sarcolemma' isolated from skeletal and cardiac muscle by a variety of techniques and reported by a number of different authors [19,20,25]. All these values are significantly lower than those

obtained in this laboratory and those reported by Andrew and Appel [1] and by Festoff and Engel [3] for light membranes isolated by LiBr-KCl extraction procedures followed by density gradient purification. Since treatment with high salt concentrations (usually of $Na^+ + I^-$) is often used in the preparation of 'purified' ($Na^+ + K^+$)-ATPase [36] and presumably affects this purification in part by removal of less tightly bound membrane proteins, the possibility must be considered that LiBr membranes are partially stripped of membrane protein resulting in higher observed specific activities for intrinsic proteins such as the ($Na^+ + K^+$)-ATPase. This explanation seems unlikely, however, since treatment of LSS membranes with LiBr subsequent to isolation fails to yield specific activities of *p*-nitrophenylphosphatase comparable to that obtainable with the LiBr isolation procedure.

Reproducible differences also exist in cholesterol content between these two light membrane fractions. Relatively low values of cholesterol similar to those seen here with LSS-light membranes have been reported by other authors for muscle 'sarcolemma' [4]; on the other hand high values similar to those which we observed for LiBr light membranes are also found in the literature [2,21]. It should be noted that both sets of values are significantly higher than those reported for sarcoplasmic reticulum vesicles purified by sucrose density centrifugation (5—15 μg cholesterol/mg protein), and many workers feel that true sarcoplasmic reticulum vesicles contain no cholesterol [37]. Studies with digitonin perturbation of cholesterol and Ca^{2+}-ATPase distributions on density gradients support this contention [38], and an analogous situation is reported in liver where surface membrane contains high cholesterol levels but purified endoplasmic reticulum appears to be virtually cholesterol free [39].

It is unlikely that the observed differences in cholesterol content could produce the difference noted in [^{3}H]saxitoxin binding or in acetylcholine receptor density, since for both receptors assays were carried out with detergent-solubilized membrane proteins. Under these conditions, the local membrane environment which could be modified by changes in cholesterol level is destroyed. Similarly, differences in SDS-polyacrylamide gel profiles of membrane proteins cannot be secondary to differences in cholesterol content. The possibility does remain, however, that lower *p*-nitrophenylphosphatase levels observed in LSS-light membranes reflect the lower cholesterol content in that membrane rather than a lower level of enzyme protein.

The binding of [^{3}H]saxitoxin to rat muscle homogenate suggests a density of sites (approx 15 pmol/g wet wt.), similar in magnitude to that previously reported in frog skeletal muscle (22 pmol/g wet wt. [40]) and in rat diaphragm (24 pmol/g wet wt. [41]). The observed binding constants for saxitoxin in the isolated surface membrane preparations and the competition by tetrodotoxin agree well with comparable physiologic data in mammalian muscle suggesting that the identification of specific saxitoxin binding sites as sodium channels is valid in these preparations. The calculated value for the density of sodium channels per mg membrane protein in the LiBr-light membrane preparation is comparable to reported values for other excitable membranes which have been isolated in purified form (see Table II and refs. 42—44). The 56-fold enrichment of saxitoxin binding seen in the muscle membrane fraction when compared to crude muscle homogenate is considerably in excess of the usual 2—4-

fold increase over homogenate which we find in purified synaptosomes from rat brain [13]. The homogeneous distribution of saxitoxin binding sites within the LiBr fraction favors a fairly uniform membrane population, an observation which must be weighed against the slight apparent inhomogeneity of *p*-nitrophenylphosphatase distribution.

The lower density of sodium channels in LSS-light membranes may be the result of several factors. First, this fraction may be composed predominantly of T-system membranes and a lower site density would be expected from available physiological data [33]. Second, the work of Lau et al. [10] suggests that some portion of the T-system tends to form vesicles with their cytoplasmic surfaces outward; in such vesicles the binding sites for saxitoxin, although present, would not be accessible and hence would not be detected by our methods.

In our study this possibility has been ruled out on the basis of [^{3}H]saxitoxin binding measurements in solubilized membrane which yield results comparable to those in intact vesicles. The presence of significant populations of inside-out vesicles cannot be contributing to our reported results. A similar argument holds for measurements of acetylcholine receptor density since here, too, determinations were carried out on solubilized membrane material. These conclusions regarding vesicle-sidedness are supported for the LSS-light membrane by our earlier studies indicating that nearly all vesicles in this fraction bind ferritin-labeled concanavalin A to their outer surfaces, as demonstrated by electron microscopy [6].

It is of interest that the apparent K_d for saxitoxin is unchanged in either LSS- or LiBr-light membranes fractions when compared to homogenate. It appears that at least this intrinsic membrane protein is not significantly modified in this respect by the prolonged salt extraction required for the LiBr preparation.

The results of the present study suggest that caution must be exercised in comparing results of different studies reported in the literature on muscle 'sarcolemma' since it appears that varying amounts of true sarcolemma and T-tubular membranes may be co-isolated in each case depending on the procedure used. Since chemical properties and densities of specific functional proteins vary between these membranes, significant variations in average values between methods and between different systems in which the same method has been applied can be anticipated which might not reflect actual true differences in sarcolemmal characteristics. In each system a combination of various estimators for sarcolemma must first be applied to establish the apparent level of purity, and only preparations of similar purity can be reasonably compared. We presently feel that the LiBr-light membrane preparation described here, which is similar to those reported by several other authors, represents the closest approximation available to pure sarcolemma.

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