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In Vitro Analysis of the General Properties and Junctional Receptor Characteristics of Skeletal Muscle Membranes. Isolation, Purification, and Partial Characterization of Sarcolemmal Fragments*

(membrane ATPase/bungarotoxin/acetylcholinesterase/cholinergic/nicotinic)

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ABSTRACT **Muscle membranes were partially purified from rat leg muscles. Externally oriented membrane functions were used to monitor and characterize the resulting membrane fractions. Na^+K^+-stimulated Mg^{++}-adenosinetriphosphatase, acetylcholinesterase, and cholinergic receptor activities are present and enriched in the density-gradient subfractions of crude sarcolemma when compared with the first pellet. The physical separation of the cholinesterase and receptor activities on the gradient subfractions is demonstrated.**

Receptor activity, determined by specific ^{125}I-labeled alpha-bungarotoxin binding, appears in fractions with densities similar to other plasma membranes (D_4^{20} 1.1015–1.1520). Acetylcholinesterase, on the other hand, is preferentially distributed in lighter density fractions (D_4^{20} 1.0507–1.0780) and parallels the gradient distribution of the ATPase. In sodium dodecyl sulfate–polyacrylamide gel electrophoresis, a high-molecular-weight glycoprotein sediments with the higher density fractions only.

The data suggest a molecular dissection of the layers of the sarcolemma. The receptor is tentatively felt to be an integral component of the junctional plasma membrane. Acetylcholinesterase is felt to be superficially located on the ectolamina of the junctional sarcolemma, and may be woven within the matrix of the intersynaptic basement membrane.

Muscle membranes possess properties found in the membranes of all living cells as well as those unique to excitable tissues. Muscle, however, has not been extensively used as a model system for evaluating new concepts and methodology of membrane molecular biology. Its relative resistance to cell disruption, extensive connective tissue network, and the multiple layers of the sarcolemma may partially account for this. For the purposes of this paper, the sarcolemma is defined as a multilayered envelope covering the muscle cell, including, as its inner sheath, the trilaminar plasmalemma (true plasma membrane) external to which is a basement membrane, also composed of several layers. Attempts have been made to isolate "purified" skeletal muscle sarcolemma, with varying degrees of success (1–6). We have used a modification of a method used to prepare amphibian muscle membrane (3) for our work on a mammalian sarcolemma.

Abbreviations: ACh, acetylcholine; AChE, acetylcholinesterase (EC 3.1.1.7); AChR, acetylcholine receptor; NaKATPase, Na^+K^+-stimulated, Mg^{++} ATPase (EC 3.6.1.3); SDS, sodium dodecyl sulfate; SLF, sarcolemmal membrane fragments; αBT, alpha-bungarotoxin; DFP, diisopropylfluorophosphate; PAS, periodic-acid Schiff reagent.

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Various "markers" have been used to determine the relative purity of plasma membranes derived from cell homogenates (7, 8). In addition to Na^+K^+-stimulated Mg^+ ATPase (EC 3.6.1.3; adenosinetriphosphatase) (NaKATPase), we have chosen to monitor two other functions thought to be sarcolemmal in nature. Acetylcholinesterase (EC 3.1.1.7) (AChE) and acetylcholine receptor (AChR) activities, and the membrane protein patterns on Na dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis, have been employed to characterize the membrane fragments thus isolated.

Our results demonstrate that sarcolemmal membranes can be prepared relatively free of contaminating mitochondria, myofibrillar material, and sarcoplasmic reticulum. The functional components of these sarcolemmal fragments are enriched in activity compared to the first pellet. These activities can be physically separated on the gradients and may, therefore, represent the dissociated molecular constituents of the several sarcolemmal layers. The physical separation of cholinergic functions should prove a useful tool for the study of the sarcolemma *in vitro* as well as excitable membranes in general.

MATERIALS AND METHODS

Preparation of Sarcolemmal Membrane Fragments (SLF). A modification of the method of Boegman *et al.* (3) was used to isolate fragments from male Sprague–Dawley rats (200–300 g) killed by cervical dislocation. Gastrocnemius-plantaris muscles (mixed fiber types) were rapidly dissected and placed in ice-cold sucrose medium (0.25 M sucrose; 0.2 mM Na_2-EDTA, 0.1 M Tris·HCl buffer, pH 7.6). Large nerves, blood vessels, and most of the connective tissue were removed. Muscles were homogenized (5 volumes of sucrose medium) in a Virtis 45 homogenizer at setting 60 for 1.5 min, and rehomogenized (2 volumes of sucrose medium) at setting 40 for 1 min. All subsequent steps were at 0–4°. The salt extraction in LiBr and KCl as well as differential centrifugation were as described (3).

The final pellet, $P_{10s}H_2O$ (10–20 mg/ml of protein in 0.9 ml) was layered on a sucrose gradient and centrifuged at 200,000 $\times$ g_{max} for 3.5 hr. Initially, a continuous (15–45% sucrose) gradient was used. In later experiments, discontinuous gradients (0.4–1.4 M sucrose in 0.2 M steps) were routinely used.

Assays. Protein was estimated by the method of Lowry *et al.* (9), with bovine-serum albumin as standard.

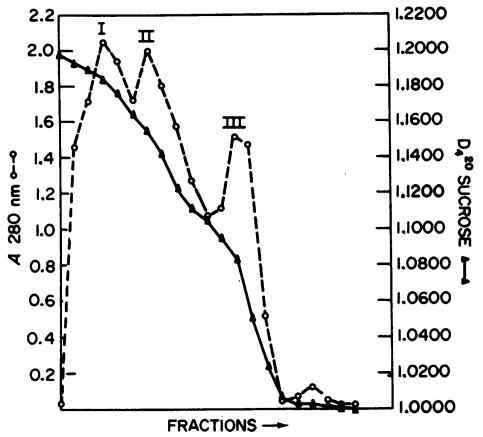

FIG. 1. Representative continuous sucrose gradient preparation: 15–45% sucrose in 0.5 mM H_4EDTA, 4.0 mM Tris·HCl, pH 8.4. Centrifugation was performed as described in *Methods*.

Modifications of the methods of Post and Sen (10) and of Wahler and Wollenberger (11) were used to assay ATPase. Typical assay conditions were 35–50 μg of membrane protein, 3 mM MgATP, 100 mM NaCl, 20 mM KCl, 7.5 mM $MgCl_2$, pH 7.5, 37°. Specific activity = μmoles of P_i hr^{-1}·mg of $protein^{-1}$.

A modification of the radiochemical AChE assay of Potter (12) was used ([^{14}C]acetylcholine; Amersham Searle, specific activity 10 mCi/mmole).

Receptor Binding Assay. Crude *Bungarus multicinctus* venom (Miami Serpentarium) was fractionated (13), and fraction toxicity was determined in white mice. Neuromuscular blocking activity was assayed in a frog rectus abdominis preparation. Alpha-bungarotoxin (αBT) was iodinated (carrier-free Na^{125}I; Amersham-Searle) by modifications of the Chloramine T (14) or insoluble lactoperoxidase methods (15). The specific activity of the iodinated toxin was 2–4 Ci/mmole.

SLF subfractions (50–100 μg of protein) were incubated in 0.01 M Na-phosphate buffer, pH 7.4 (final volume of 150 μl) at various temperatures. In competitive studies, ligands were preincubated with SLF for 30 min. To initiate binding, 0.05 nmoles of ^{125}I-labeled αBT were added. To terminate binding, 100-μl aliquots were pipetted into tubes containing 3 ml of ice-cold wash solution (0.15 M NaCl in 0.01 M Na-phosphate buffer, pH 7.4) and filtered by gravity or under mild vacuum, through 1–2 anion-exchange filter paper discs (Whatman, DE81) previously equilibrated in buffer. Salt was added to remove any nonspecific adsorption of toxin. The discs were washed with wash solution, under suction, in a Millipore filter assembly four times, air-dried, and counted with a NaI crystal (Picker Liquimat).

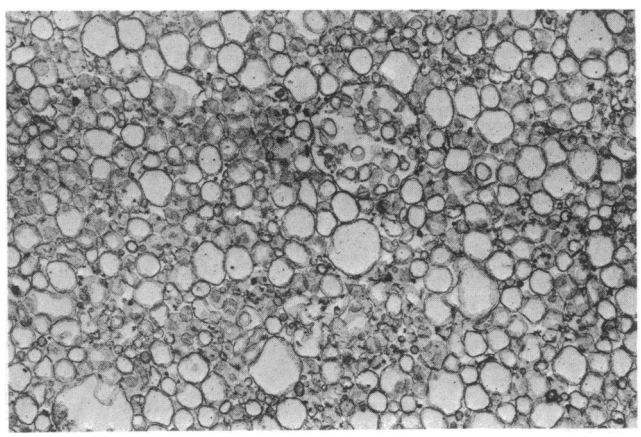

FIG. 2. Electron micrograph of discontinuous gradient subfraction, band 4 (× 10,810), isolated as described in *Methods*.

TABLE 1. *ATPase activity. Ionic stimulation and ouabain inhibition of $P_{105}H_2O$ and discontinuous gradient subfractions*

	$P_{105}H_2O$	Band 1	2	3	4	5	Pellet
Ca^{++}	6.6 ± 0.7	20.6 ± 0.8	7.8 ± 1.0	7.8 ± 0.6	10.0 ± 0.7	4.8 ± 0.4	3.2 ± 0.6
Mg^{++}	7.2 ± 1.3	23.1 ± 1.0	5.6 ± 0.6	5.4 ± 1.1	10.2 ± 0.6	5.0 ± 0.6	2.8 ± 0.5
Mg^{++} and ouabain	10.98 ± 1.8	18.2 ± 0.7	7.8 ± 1.7	5.0 ± 0.8	10.8 ± 1.0	3.3 ± 0.2	2.9 ± 0.4
NaKMg	33.54 ± 2.6	48.9 ± 0.4	3.6 ± 0.5	6.8 ± 0.5	14.4 ± 1.1	5.7 ± 1.0	2.4 ± 0.4
NaKMg and ouabain	12.21 ± 1.4	20.3 ± 0.9	9.2 ± 1.0	4.4 ± 1.0	4.0 ± 1.1	5.6 ± 0.7	2.6 ± 0.3
No added ions	10.98 ± 0.9	23.4 ± 0.6	7.8 ± 1.0	5.6 ± 0.9	5.6 ± 0.9	4.6 ± 0.7	3.0 ± 0.8

ATPase specific activities of discontinuous gradient subfractions under influence of various cations and cardiac glycosides (ouabain, 10^{-4} M), as described in *Methods*. Values are means of four different preparations ± SEM (specific activity as in *Methods*).

SDS-Polyacrylamide Gel Electrophoresis. A modification of the method of Fairbanks *et al.* (16) was used. Protein (20–40 μg), in sample solution, was incubated, in the presence of 4 mM dithiothreitol (Sigma), at 56° for 20 min. Solubilization was complete under these conditions. Proteins were stained with Coomassie blue (16) and glycoproteins with periodic acid-Schiff reagent (PAS) (17).

Molecular weight standards used were: insulin (5700), ribonuclease (13,700), pepsin (36,000), human-serum albumin (69,000), and phosphorylase *a* (93,000). Gels were photographed and scanned (Gilford model 2410-S linear transport attachment; 550 nm for Coomassie blue, 560 nm for PAS).

Electron Microscopy. Membrane samples were fixed for 2 hr at 4° in modified Karnovsky fixative (18), centrifuged at 100,000 × *g* into tightly adhering pellets, washed in buffer (pH 7.4), and post-fixed (1 hr at 4°) in 1% osmium tetroxide. After dehydration in graded methanol solutions in propylene oxide, the tissue was embedded in Spurr low-viscosity embedding media, thin-sectioned, stained with uranyl acetate–lead citrate, and examined with an AEI EM6B electron microscope.

RESULTS

Membrane Isolation. A prerequisite in a muscle-membrane isolation scheme is an effective method for removal of myofibrillar and other sarcoplasmic protein. Techniques for extruding cytoplasm from sarcolemmal tubules (1, 2, 4–6) had significant contamination with actin and myosin. Our method and results are similar to those of Boegman *et al.* (3) in the preparation of vesicular sarcolemmal fragments from frog leg muscle. Of the original pellet (P_9) protein, 65–70% was removed in LiBr and KCl extraction steps. Less than 1% of P_9 protein was present in the "crude membranes" ($P_{105}H_2O$). This protein was largely myofibrillar by chemical assays (unpublished).

Further purification is achieved by density gradient centrifugation, either continuous or discontinuous. The absorbancy profile for a representative continuous gradient preparation is shown in Fig. 1. Three protein peaks, labeled from the bottom of the gradient I, II, and III, in addition to a

TABLE 2. *Ionic stimulation of sarcolemmal and sarcoplasmic reticular membranes*

	No added ions	Ca^{++}	Mg^{++}	Mg^{++} and ouabain	Na^+, K^+, Mg^{++}	Na^+, K^+, Mg^{++}, ouabain
DG Band 1	23.4 ± 0.6	20.6 ± 0.8	23.1 ± 1.0	18.2 ± 0.7	48.9 ± 0.4	20.3 ± 0.9
SVF	7.0 ± 0.4	9.2 ± 0.4	10.1 ± 0.1	10.2 ± 0.3	11.0 ± 0.1	4.9 ± 0.6

Comparison of discontinuous gradient (*DG*) subfraction (band 1) NaKATPase specific activity with a sarcoplasmic vesicular fraction (*SVF*). Isolation and enzyme assays as described in *Methods*. Values are specific activity means of three different experiments ± SEM.

pellet, were usually obtained. Discontinuous gradients routinely gave five bands plus a pellet.

Numerous large and small vesicles, free of mitochondria and osmiophilic staining material were present in electron micrographs of peaks II and III of continuous, and bands 1–4 (Fig. 2) of discontinuous preparations. Vesicle fragments were also present in the more dense fractions, but contamination with mitochondria and myofibrillar material was great.

ATPase Distribution in SLF. The highest specific activities were found in the less dense fractions in both preparations [i.e., peak III of the continuous gradient, 29.4 specific activity units (D_4^{20} 1.0520–1.0920) and bands 1 and 2 of the discontinuous gradient preparations (D_4^{20} 1.0507–1.0780), 48.9 specific activity units]. NaKATPase enrichment for discontinuous gradient band 1 relative to the first pellet was 4-fold. However, ouabain inhibition could not be obtained in P_9, and, therefore, this "purification" may be underestimated by as much as 10-fold. Subsequent experiments were performed on SLF subfractions isolated by discontinuous gradient centrifugation.

Table 1 shows the effects of various ions and ouabain, to distinguish the several known muscle ATPases (1–6, 19). When compared with tubes containing no added ions (EDTA + deionized water) several observations can be made. First, there is no Ca^{++} stimulation in the lighter fractions. Second, both Ca^{++} inhibition (60%) and ouabain inhibition is greatest in the lighter subfractions. Third, some Ca^{++} stimulation occurs in band 4.

We next compared the ionic stimulation of ATPase activities of SLF band 1 and a fraction thought to represent sarcoplasmic reticulum (Table 2). The sarcoplasmic vesicular fraction is stimulated by Mg^{++} alone and to a lesser extent, by Ca^{++}. Ouabain (10^{-4} M) inhibition and $Na^+ + K^+$-stimulation of ATP hydrolysis are also present in this fraction.

AChE Distribution. Fig. 3 shows the gradient distribution of AChE, representing the specialized junctional area of the muscle membrane. DFP and eserine (physostigmine) were used to separate the specific junctional AChE from other esterases (20). All activity subsequent to "crude membranes" ($P_{105}H_2O$) is due to cholinesterase, since eserine (10^{-5} M) completely inhibits. Before this step, 20–30% of ACh hydrolysis is not inhibited by this concentration of eserine. The highest specific activity for AChE (not inhibited by 10^{-8} M DFP) is found in band 1. Significant activity is also found in band 5 (D_4^{20} = 1.1560–1.1856). AChE can be found distributed among several gradient fractions (21) and appears to distribute in a bimodal fashion in the subfractionation

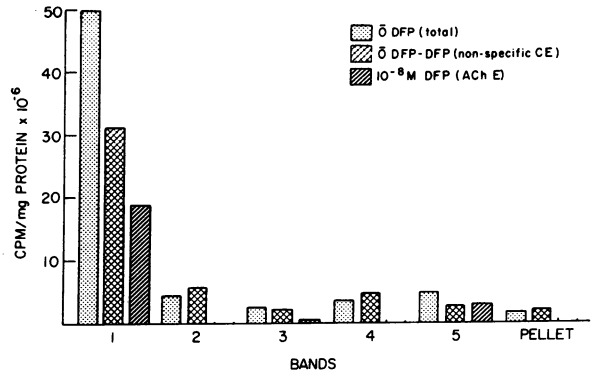

FIG. 3. Distribution of specific, junctional AChE on discontinuous sucrose gradient as described in *Methods*. AChE activity represents non-DFP (10^{-8} M)-inhibited activity. Values given are means of 5 determinations, standard error being less than 5%. *CE*, cholinesterase. Values for cpm per mg of protein have been multiplied by 10^{-6}, as indicated.

of these "crude sarcolemmal membranes." Partial "solubilization" of certain enzyme activities such as AChE may occur with vigorous treatment (22), causing them to distribute in lighter fractions. However, the eserine-inhibitable activity sediments at 105,000 × *g* and continues this association through two H_2O washes. Since our banding densities for AChE are considerably lighter than those of other plasma membranes (7), the data may be interpreted to indicate that much of the AChE at the mammalian neuromuscular junction may reside in other than the plasma membrane (see below).

The SLF-band 1 specific activities for AChE by the radiochemical assay compare with those reported by Potter for rat-brain cortex (12) and are several orders of magnitude lower than purified electric eel AChE (our unpublished results). The recovery of specific AChE is difficult to assess since before the gradient input, significant activity escapes eserine (10^{-5} M) inhibition. An enrichment of 25-fold is present in the gradient input and 110-fold in gradient band 1 relative to the first pellet.

AChR Distribution. The binding of ^{125}I-labeled αBT to SLF membrane particles was used to determine junctional membrane AChR activity. Several methods (23–25) were initially used. With the high pK_a (9.2) of αBT (26) and the more acidic pK of the receptor (25, 27), use of the DE81 filters (after they were adequately washed with 0.15 M NaCl) gave linear binding with increasing amounts of "crude membrane" or SLF protein. Differences were seen depending on the incubation temperature. Binding was linear to 10 min, and essentially complete at 30 min. Addition of "carrier" protein (such as bovine-serum albumin) did not appreciably alter the binding.†

The binding to SLF fractions of ^{125}I-labeled αBT specific for nicotinic cholinergic receptor sites is shown in Table 3. Nicotinic cholinergic ligands (10^{-4} M), protected against ^{125}I-labeled αBT binding to varying degrees, while at the same concentration atropine, a potent muscarinic antagonist, was without effect. In no experiment could greater than 78% blockade of binding sites be achieved (23–25, 28).

† Festoff, B. W. & Engel, W. K., manuscript in preparation.

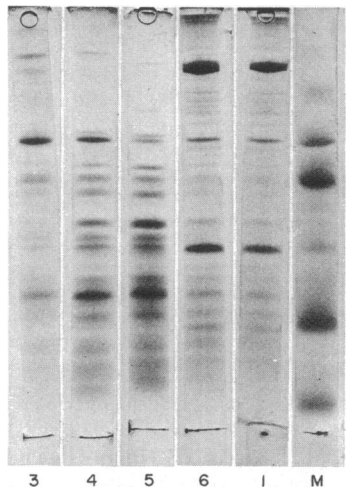

FIG. 4. SDS gel electrophoresis of discontinuous gradient fractions. Bands 1 + 2 contained too little protein to be visualized photographically. *I*, input to gradient ("crude membranes"); *M*, marker mix. Electrophoresis and staining as described in *Methods*.

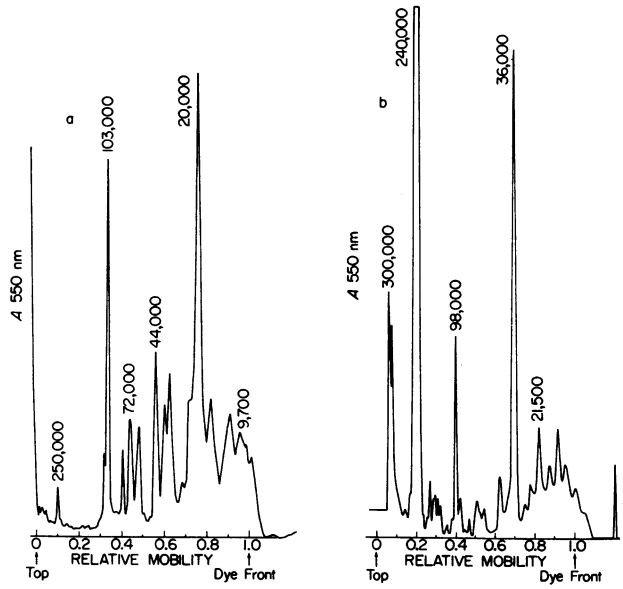

FIG. 5. SDS–polyacrylamide gel electrophoresis of middle and higher density subfractions of discontinuous gradient preparations, as described in *Methods*. (*a*) Band 3; (*b*) band 5. Scale is the same for both gels.

Total recovery of toxin-binding material relative to the first pellet is about 8%. However, considerable purification of nicotinic-competing specific activity occurs (150-fold enhancement in discontinuous gradient band 4 compared to P_9). The number of receptor sites per endplate has been estimated. For mammalian diaphragm endplates, using iodinated αBT, this figure is about 1 to 5 $\times 10^7$ sites (24, 28, 29). Though the number of fibers in rat gastrocnemius-plantaris is not known, the medial gastrocnemius of guinea pig (R. Edgerton, personal communication) contains approximately 14,000. Assuming the equal weight of the lateral head and about $^1/_3$ the weight for plantaris, then the total number of singly innervated fibers, and hence, endplates, would be about 33,000. Using these estimates and our specific binding data of 3.5 pmoles of αBT per muscle group, we arrive at 6.6 $\times 10^7$ binding sites per endplate in these rat leg muscles.

Contrary to our findings on ATPase and AChE distribution, the highest binding of ^{125}I-labeled αBT per mg of protein (specific for nicotinic sites) occurred in bands 3 and 4 (D_4^{20}, 1.1015–1.1520) (Table 3). Apparent nonnicotinic binding was present in band 5. These banding densities are within the range accepted for plasma membranes (7).

SDS-Polyacrylamide Gel Electrophoresis. Highly reproducible protein patterns are obtained from "crude membranes" and SLF subfractions. A representative photograph of $P_{105}H_2O$ and subsequent discontinuous gradient separation of SLF is shown in Fig. 4. Certain striking differences in the profiles are evident. In the heaviest fractions, a deeply stained, high-molecular-weight protein (about 240,000) correlates with a similar band in the input ($P_{105}H_2O$). It is less evident in the lighter density fractions.

Further differences of light and heavy fractions are seen when they are scanned at 550 nm (Fig. 5). In lighter fractions (Fig. 5*a*) the most prominent bands are 20,000, 44,000, 72,000, and 103,000 daltons. In contrast, in the heaviest gradient fractions (Fig. 5*b*), the most prominent bands are at 36,000, 98,000, and the previously mentioned 240,000-dalton protein. A single, deeply stained glycoprotein was seen in band 5 and the gradient pellet, and correlated with the unique protein band at 240,000 molecular weight. In band 3 and occasionally in 4 a light-stained PAS glycoprotein with an apparent molecular weight of 98,000 was seen.

DISCUSSION

The sarcolemmal membrane possesses characteristics ideally suited for the study of the excitable tissue. Intercellular communication and the effects of neurotransmitters, hormones, and drugs on membrane functions may be monitored. Its specialized "endplate" region is a "simplified" synapse and has been extensively studied with electrophysiological techniques. The subcellular characterization of both the general muscle membrane and its specialized junctional receptor area should logically follow.

Inherent in muscle membrane purification schemes is the need to remove myofibrillar material. In our present studies, successive salt extraction with concentrated LiBr and KCl followed by sucrose gradient centrifugation greatly eliminates

TABLE 3. *Specific binding of ^{125}I-labeled αBT to "crude" and density gradient-purified muscle membranes*

		dtc		CCh		Atropine	
	Control	10^{-4} M	% Inhib.	10^{-4} M	% Inhib.	10^{-4} M	% Inhib
$P_{105}H_2O$	0.045 ± 0.009	0.012 ± 0.004	73	0.010 ± 0.008	78	0.040 ± 0.010	11
DG Band 1	0.014 ± 0.001	0.006 ± 0.001	57.1	0.004 ± 0.001	71	0.013 ± 0.003	7
2	0.033 ± 0.009	0.020 ± 0.001	39.3	0.015 ± 0.004	54.5	0.033 ± 0.004	0
3	0.153 ± 0.005	0.050 ± 0.009	67.3	0.062 ± 0.006	59.4	0.161 ± 0.008	0
4	1.25 ± 0.009	0.384 ± 0.009	69.3	0.388 ± 0.005	69.0	1.23 ± 0.010	1.6
5	0.315 ± 0.004	0.290 ± 0.006	7.9	0.270 ± 0.010	14.3	0.306 ± 0.010	2.9
Pellet	0.045 ± 0.007	0.039 ± 0.010	13.3	0.033 ± 0.011	26.7	0.042 ± 0.012	6.7

Binding of ^{125}I-labeled αBT to input and subfractions of discontinuous gradient (*DG*) preparations using DE81 filter disc assay as described in *Methods*. Cholinergic ligands were preincubated with membranes for 30 min before addition of labeled toxin. Values represent specific activity (cpm $\times 10^{-6}$/mg of membrane protein) means of three determinations of four different preparations ± SEM. *dtc*, *d*-tubocurarine; *CCh*, carbachol.

contaminating myofibrillar, as well as sarcoplasmic reticular, material. The resulting SLF subfractions are enriched for NaKATPase, AChE, and AChR activities, all believed to be external-membrane-oriented functions in skeletal muscle.

Of great interest is our demonstration of disparate particulate localization of AChE and AChR of mammalian sarcolemmal membranes on discontinuous sucrose gradients. The separation of AChE-enriched (and indirectly, AChR function) from ATPase-enriched particles in electric eel membranes has been described (29). Subsequently, separation of AChE from AChR-containing particles of sonicated *Torpedo marmorata* electric tissue by discontinuous (31) or continuous (32) gradients was demonstrated.

Some recent studies suggest that the AChE and AChR may also have different locations *in vivo*. Selective removal of AChE from mouse diaphragm neuromuscular junctions with collagenase treatment has been shown, while pre- and postjunctional membrane structure and electrical activity were preserved (33). AChE was thought to be bound in the intersynaptic matrix or to reside, superficially, on either the nerve terminal or muscle membrane. It has also been suggested (34) that AChE is anchored to the ectolamina of the basement membrane, where it is easily removed with mild proteolytic enzyme treatment. Evidence (35, and refs. therein) suggests that the basement laminae of muscle cells are composed of inner and outer sheaths both external to the plasma membrane, which are quite different in their physicochemical character. Glycoprotein–mucopolysaccharide staining and binding of colloidal iron have been demonstrated on the ectolamina. In our studies, a unique high-molecular-weight (240,000) glycoprotein is found in the heaviest gradient fractions, which also contain AChE activity. It is possible that this fraction is largely composed of dense material making up the basement membrane. The AChE activity in the lightest subfraction may represent the superficially bound enzyme easily released from the intersynaptic matrix.

Our results indicate that mammalian skeletal muscle can be homogenized to prepare particles enriched for external membrane functions, essentially free of contaminating myofibrillar material. The method appears generally applicable for preparation of sarcolemmal membranes. Relatively little sarcoplasmic reticular activity is present in the final purified particles. Loss of SLF material into initial "soluble" fractions may occur. Our experiments have shown NaK-stimulated, ouabain-inhibited ATPase activity but little or no ^{125}I-labeled αBT-binding or AChE activity (unpublished results) in the initial low-speed supernatant. This activity may reflect T-tubule membrane containing external general, but not junctional, membrane functions.

The studies of excitable membranes at the physiological level prompts us to pursue the use of SLF subfractions as an *in vitro* system for the microphysiologic and molecular study of mammalian neuromuscular events. The techniques established in these studies may prove useful in our approach to the study of possible membrane defects in human neuromuscular disorders.

Note Added in Proof. Since this manuscript was in preparation, separation of AChE from AChR-containing particles using continuous gradients has been reported in *Torpedo californica* [Duguid, J. R. & Raftery, M. A. (1973) *Biochemistry* **12**, 3593–3597.]

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