Isolation of Plasma Membrane Vesicles from Rabbit Skeletal Muscle and Their Use in Ion Transport Studies*

Steven Seiler‡ and Sidney Fleischer
From the Department of Molecular Biology, Vanderbilt University, Nashville, Tennessee 37235

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A method has been developed for the isolation of sealed plasma membrane vesicles from rabbit white skeletal muscle. The final preparation was highly purified as indicated by enrichment of plasma membrane marker enzymes (i.e. ouabain-sensitive (Na⁺,K⁺)-ATPase, adenylate cyclase, and acetylcholinesterase). The absence of sarcoplasmic reticulum and mitochondria as contaminants was indicated by the low specific activity of marker enzymes, i.e. Ca²⁺-ATPase, succinate-cytochrome c reductase, and monoamine oxidase. Thin section and negative staining electron microscopy confirmed the absence of sarcoplasmic reticulum and mitochondrial contamination.

The plasma membrane preparation consisted largely of sealed vesicles as observed by electron microscopy and as also demonstrated by latency of enzymic activities, which were unmasked by preincubation with detergent (sodium dodecyl sulfate). Membrane sidedness was estimated from latency of ouabain-sensitive (Na⁺,K⁺)-ATPase activity and acetylcholinesterase activity. The latency studies suggest that most of the vesicles are oriented inside out with respect to the orientation of the sarcolemma membrane in the muscle fiber.

The inside-out plasma membrane vesicles actively accumulated sodium ions upon addition of ATP. The sodium ions were concentrated greater than 8-fold inside the vesicles and were released upon addition of the ionophore monensin. The sodium ions were taken up in the presence of K⁺ or NH₄⁺ but not of choline. Uptake was inhibited by low concentrations of vanadate or digitoxin. The Na⁺ uptake was concomitant with Rb⁺ efflux. Therefore, the sodium ion space and the resulting gradients formed appear to have been generated by the ouabain-sensitive (Na⁺,K⁺)-ATPase. Batrachotoxin, which opens Na⁺ channels in excitable tissues, prevents most of the Na⁺ uptake, suggesting the presence of toxin-activated Na⁺ channels in these plasma membrane vesicles.

Skeletal muscle plasma membrane generates ionic gradients which are essential for excitability function. Maintenance of these gradients is provided by vectorial transport of Na⁺ outward and K⁺ inward across the plasma membrane. The transport of these ions is mediated by the (Na⁺,K⁺)-pump also referred to as the (Na⁺,K⁺)-ATPase. The resulting downhill, inwardly directed, Na⁺ electrochemical gradient is also utilized to drive uptake of amino acids (1) as well as the countertransport of Ca²⁺ via a Na⁺/Ca²⁺ exchange mechanism (reviewed in Ref. 2).

The availability of a purified plasma membrane vesicle system makes possible the study of plasma membrane composition and function uncomplicated by the presence of other organelles. The enrichment of fractions in plasma membrane from skeletal muscle has been carried out in a number of laboratories using a variety of approaches (3–17). This is the first report of the isolation of skeletal muscle plasma membrane as vesicles which measures vesicle integrity and membrane sidedness and uses such vesicles for transport studies. A preliminary report of this work has already appeared.†

**EXPERIMENTAL PROCEDURES**

Materials—Radioactive [*C]tryptamine bis-succinate, [*Na, 86Rb, and [*Ca were obtained from New England Nuclear. Sodium dodecyl sulfate that was used for sample preincubations before assay was a specially purified grade obtained from BDH Chemicals Ltd. (Poole, England). SDS† that was used to stop reactions was obtained from Fisher Scientific (Pittsburgh, PA), and imidazole (A grade), HEPES (ULTROL grade), and the ionophores A23187 and monensin were obtained from Calbiochem-Behring. Dextran T-10 was obtained from Pharmacia (Uppsala, Sweden) and was made as a stock solution (30% w/w), filtered through a 0.45-μm Millipore filter (Bedford, MA), and stored frozen until used. Density gradient grade sucrose was obtained from EM Laboratories (Elmsford, NY) or Schwarz/Mann. Ouabain, acetylcholinolamine, valinomycin, digitoxin, phosphocreatine (di-Tris salt), rabbit muscle cresteine phosphokinase, and rabbit muscle adenylic kinase were obtained from Sigma. Glutaraldehyde was obtained from Polysciences, Inc. (Warrington, PA).

Initially, both the sodium and Tris salts of adenine 5'-triphosphate (both substantially vanadium-free) were obtained from Sigma Chemical. In later studies, Tris/ATP was made from the Na₂ATP (Sigma) by passing it over a cation exchange resin equilibrated with Tris (AG 50W-X8, 100–200 mesh, from Bio-Rad). The ATP was kept as a stock solution of 0.2–0.3 M, pH 7.0–7.2, the concentration of which was determined spectrophotometrically (molar extinction coefficient at 260 nm was 1.54 × 10⁴ liters/mol·cm).

Batrachotoxin was the kind gift of Dr. John Daly, National Institutes of Health (Bethesda, MD). It was made up as a stock solution of 5 mM in absolute ethanol and diluted into the assay media to the desired concentration.

Methods—Protein was measured according to the method of Lowry et al. (33) using bovine serum albumin as a standard. Phosphorus analysis of membrane fractions was carried out as described in Rouser and Fleischer (34). Succrose and dextran T-10 concentrations were

† Seiler, S. (1981) Fed. Proc. 40, 1624.

‡ Present address, Department of Physiology, Vanderbilt School of Medicine, Nashville, Tennessee 37235.

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‡‡ Present address, Department of Physiology, Vanderbilt School of Medicine, Nashville, Tennessee 37235.

The abbreviations used were: SDS, sodium dodecyl sulfate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; SR, sarcoplasmic reticulum; PMs, plasma membrane fraction obtained from succrose gradient; PMd, plasma membrane fraction obtained from dextran T-10 gradient.
adjusted using an Abbé 3L refractometer, Bausch and Lomb. All pH adjustments were made at room temperature.

Electron microscopy was performed as previously described (26, 27).

SDS-polyacrylamide gel electrophoresis was performed using a modification of the method of LeBlanc and Beyer (28).

(Na+,K+)-ATPase—Sealed vesicles were reconstituted with detergent-free Na+,K+ ATPase activity (18, 19). The preincubation medium contained 1 mg/ml of sample protein, 40 mM imidazole/HEPES, pH 7.3, 2 mM Tris EDTA, and from 0-0.5 mg/ml of SDS. The sequence of addition was (zero time point) to determine the amount of Na+ in the vesicles prior to the addition of ATP. Active transport was initiated by the addition of ATP. The reaction proceeded for 5-10 min at 37 °C before being stopped by the addition of 0.5% (w/v) SDS, 10 mM Na3EDTA and immediate placement of the activity with and without ouabain.

Acetylcholinesterase—These membranes were extracted with SDS before assaying acetylcholinesterase activity as described (24). All pH measurements were made using a Beckman model J-21 infrared pH meter. Inorganic phosphate was determined spectrophotometrically at 232 nm (extinction coefficient of 0.5 m) 21 liters/mol. cm) was used to calculate the acetylcholinesterase activity.

Monoamine Oxidase—Monoamine oxidase activity was measured by a modification of the method of Wurtman and Axelrod (23). In order to make the activity linear with respect to protein concentration, the reaction medium (500 ml final volume) contained 50 mM KPO4, pH 7.4, 2 mM EDTA, 5 mcg/ml of ice-cold 0.5 mM imidazole, bis-succinate (3 mM), 0.1 mg/ml of ionophore A23187, 2-15 µg of protein, and either 50 µM CaCl2 or 1 mM Tris/EGTA. The reaction was carried out at 25 °C for 5 min and then terminated by the addition of 0.5 ml of 5% (w/v) SDS, 10 mM Na3EDTA and placement on ice. Inorganic phosphate was determined as described earlier for the (Na+,K+)-ATPase activity.

**RESULTS**

Isolation and Characterization of the Plasma Membrane Vesicles from Skeletal Muscles—A method has been described for the isolation of plasma membrane vesicles from skeletal muscle (cf. "Methods"). The ground muscle first received a limited bleaching and washing in 0.75 M KCl. The low speed sediment was then homogenized in isotonic buffered sucrose (0.3 M) and a microsomal fraction was obtained by centrifugation. PMs was obtained by isopycnic centrifugation.
using a sucrose density step gradient (Fig. 1A, Band 2). PMs was further purified on an isopycnic, discontinuous, dextran T-10 gradient (Fig. 1B, Band 1). The yield was approximately 3 mg for PMs and 0.5–1.0 mg for PMd from 50 g of ground muscle. A typical preparation made use of three rabbits (200 g of ground muscle) which yielded 12 mg of sucrose gradient.

The characteristics of the plasmalemma fractions are summarized in Table I. The sucrose gradient considerably enriched the fraction in plasma membrane from mixed microsomes (Table I). The (Na⁺,K⁺)-ATPase was 13-fold higher than that of the microsomes, while SR and mitochondrial contamination were decreased by 8-fold and 5-fold, respectively, as judged by Ca²⁺-ATPase and succinate-cytochrome c reductase activities.

The plasma membrane fraction obtained from the dextran gradient fraction, although decreased in yield (Table I), contained still higher ouabain-sensitive (Na⁺,K⁺)-ATPase and acetylcholinesterase (1.4- and 1.8-fold higher than sucrose preparation, respectively) (Table I). The dextran purification procedure further decreased the specific activity of succinate-cytochrome c reductase, monoamine oxidase, and Ca²⁺-ATPase activities (Table I), suggesting that the dextran T-10 gradient step also separated the sealed (see below) plasma membrane vesicles from intact mitochondria, outer mitochondrial membranes, and SR, respectively. Based on the amounts of contaminant marker activities, we estimated that the dextran plasma membrane fraction contained less than 1% sarcolemmal vesicles and less than 1% mitochondria contamination (Table I). Acetylcholinesterase co-isolated with the ouabain-sensitive (Na⁺,K⁺)-ATPase activity (Table I) in accord with the observations of others (35) that the highest specific activity of acetylcholinesterase was in sarclemma-enriched fractions.

The purified skeletal muscle plasma membrane was examined by thin section electron microscopy. The fraction contained sealed membrane vesicles of irregular shape and varying in “diameter” from approximately 0.2–0.5 μm. Only a few of the vesicles had a flattened appearance characteristic of transverse tubules (Fig. 2A). Transverse tubule-like vesicles constituted only a minor percentage of the total vesicle population.

Table I: Characteristics of fractions in the purification of skeletal muscle plasma membranes

| PMs | Yield (mg protein/50 g of skeletal muscle) | µg P/mg protein | Total ATPase (μmol/mg-h) | Ouabain-sensitive ATPase (nmol/mg-min) | ATP-dependent Na⁺ uptake (capacity, nmol/mg protein) | Ca²⁺-ATPase (μmol/mg-h) | Monoamine oxidase (pmol/mg-min) | Succinate-cytochrome c reductase (nmol/mg-min) |
|-----|------------------------------------------|-----------------|-------------------------|--------------------------------------|-----------------------------------------------|------------------------|---------------------------------|------------------------------------------|
| 125 | 2.7 | 73.4 ± 4.6 (3) | 73.5 ± 11.1 (6) | 56.5 ± 9.1 (6) | 271 (3) | 0.26 <0.03 (2) | 48.2 | 3.0 |

* Activity was measured after preincubation with SDS (Fig. 5).  
* Ouabain-sensitive ATPase as obtained by subtracting the values obtained in the presence of ouabain from the total ATPase (above).  
* Ten-min time point, measuring uptake capacity at 25 °C in a medium containing 5 mM NaCl, 50 mM KCl, 5 mM MgCl₂, 30 mM imidazole, 1 mM EGTA, and 5 mM Tris/ATP.  
* Purified sarcoplasmic reticulum (36) has Ca²⁺-ATPase activity of 3.0 μmol/mg-min measured under the conditions described. This value was used to estimate the upper limit of SR contamination. The microsome fraction contained 65–70% sarcoplasmic reticulum, the sucrose gradient fraction contained 10%, and the dextran T-10-purified fraction contained less than 1% sarcoplasmic reticulum contamination.  
* No available data exist on the specific activity of monoamine oxidase activity of purified skeletal muscle outer mitochondrial membrane. The pig heart outer mitochondrial membrane has an activity of 12.8 nmol/mg-min using benzylamine as substrate (37).  
* Purified bovine heart mitochondria have a succinate-cytochrome c reductase rate of approximately 800–900 nmol/mg-min (38). Using this value, the microsome fraction and PMs contained approximately 5% and 1% mitochondria, respectively. The mitochondrial contamination was further reduced to 0.3% in the PMd plasma membrane fraction.
of submitochondrial vesicles (Fig. 2). We did not observe any muscle filaments or collagen in the purified fractions of plasmalemma, either PMs or PMd.

The protein profiles of sucrose- and dextran-purified sarcoclemma were compared by SDS-polyacrylamide gel electrophoresis (Fig. 3). A variety of polypeptides of different molecular weights were found in the plasma membrane-enriched fractions. PMd has a significantly different relative intensity of bands compared with PMs. Most notably, the 100,000- and 40,000-dalton bands were decreased and the 65,000- and 30,000-dalton components are increased. Few of the polypeptides have been identified with regard to their functional activities. This gel pattern is distinctly different and more complex than that of purified sarcoplasmic reticulum (36).

The phospholipid content in PMd was twice the protein content on a weight basis, based on the phosphorus to protein ratio (Table I), and the amount was increased over that of PMs. The cholesterol to phospholipid molar ratio was 0.39.3

Estimate of Sidedness—The estimate of sidedness is based on two assumptions. 1) Acetylcholinesterase is localized only on the outer face of the plasmalemma and is exposed in right side-out vesicles; and 2) ouabain-sensitive (Na⁺,K⁺)-ATPase cannot be measured in sealed vesicles. Ouabain-sensitive (Na⁺,K⁺)-ATPase activity should not be measurable in sealed plasma membrane vesicles regardless of orientation of the vesicles since ATP and ouabain bind on opposite membrane faces of this transmembrane pump (40, 41). In sealed right side-out vesicles, the ouabain but not the ATP binding would be accessible so that ATPase activity would not be measured (Fig. 4), whereas the opposite situation would pertain for sealed inside-out vesicles. (Na⁺,K⁺)-ATPase activity would be expressed but would not be inhibited by the ouabain.

The latent activities can be expressed by preincubation of the plasma membrane fraction with SDS (Fig. 5), which makes the vesicles leaky so that both the ouabain-sensitive (Na⁺,K⁺)-ATPase and acetylcholinesterase activities are expressed. The amount of detergent required in order to obtain maximal enzyme activity was the same for both the (Na⁺,K⁺)-ATPase and the acetylcholinesterase (Fig. 5), consistent with a common action for detergent activation, i.e. the sealed vesicles become leaky. The enzymic activity declined somewhat when excess detergent was used in the preincubation
membrane-enriched fractions. A Hoefer model SE520 0.75-mm pg of protein of sucrose gradient-enriched plasma membranes was used with an 8.75% acrylamide resolving gel and 3% stacking gel. slab gel apparatus (Hoefer Scientific Instruments, San Francisco, CA) The sample was applied to the gels after being solubilized in buffer containing 1% SDS and heating at 90 °C for 30 min. The gels were stained with Coomassie brilliant blue B) and 30,000, soybean trypsin inhibitor, 21,000, and lysozyme, bovine was concentration. The optimal SDS concentration for preincubation depended on the purity of the sample being assayed. It optimal SDS concentrations paralleled the increased lipid content. Therefore, it was desirable to optimize detergent concentration. The optimal SDS concentration for preincubation depended on the purity of the sample being assayed. It was 0.2 and 0.4 mg of SDS/ml for 1 mg/ml of membrane protein for the microsomes and the plasma membranes purified at either gradient stage, respectively. The increased optimal SDS concentrations paralleled the increased lipid content of the membrane.

The percentage of the sealed vesicles was estimated by measuring the percentage of latent activity. Approximately 71% of the ouabain-sensitive (Na⁺,K⁺)-ATPase of the sucrose gradient-enriched plasma membranes was latent, and 85% was latent in the dextran fraction (Table II). Therefore, the dextran gradient purification removed some 14% of unsealed vesicles. Acetylcholinesterase was used as an index of right side-out and leaky vesicles (Fig. 4). Approximately 34% of the acetylcholinesterase was measured in PdM without detergent treatment and, subtracting 15% leaky vesicles, we estimate that 19% were right side-out. Most of the vesicles (66%) were sealed and inside out (Table II).

The sidedness of the purified plasmalemmal vesicles was also estimated from the ouabain inactivation of the (Na⁺,K⁺)-ATPase activity, per se, assayed in the presence of the ionophore monensin (Fig. 7). Monensin renders the vesicles permeable to Na⁺ and K⁺, thereby eliminating the electrochemical gradient which would otherwise build up and retard the (Na⁺,K⁺)-ATPase activity in a sealed vesicle. When monensin-treated vesicles were pretreated with detergent, the enhanced activity (23%) estimated the sealed right side-out vesicles (Fig. 6, C versus E). The increase in the ouabain-sensitive ATPase activity by detergent pretreatment (Fig. 6, D versus F) (50%) is a measure of the sealed inside-out plasma membrane vesicles. By difference from 100%, approximately 27% of the vesicles were estimated to be leaky by this procedure.

The two methods to measure membrane sidedness (Fig. 6 and Table II and Fig. 5) gave similar results albeit with some consistent differences.

**Na⁺ Transport Studies**—Both types of plasma membrane preparations (PMs and PMd) concentrated Na⁺ upon energization with ATP (Fig. 7). The amount of Na⁺ taken up reached a plateau within approximately 12 min. The dextran-
purified fraction exhibited both a faster uptake rate and a larger capacity than PMs, consistent with the increased purity and increased amount of sealed vesicles (Tables I and II). A concentration gradient was generated which was 8-fold greater inside. This is a minimum estimate and could be closer to 10-fold when correction is made for approximately 20% right side-out vesicles.

The addition of the Na⁺ ionophore monensin (Fig. 7) caused rapid release of Na⁺ from the vesicles, confirming that Na⁺ was taken up against a concentration gradient. At the concentration used, monensin had little effect on the (Na⁺,K⁺)-ATPase activity after detergent pretreatment and, therefore, did not appear to inhibit the Na⁺ pump activity.

The effect of various other ions on the Na⁺ uptake was determined to further characterize the Na⁺-pumping activity.

NH₄⁺ and K⁺ ions facilitated the Na⁺ uptake, whereas choline did not (Fig. 8). Ammonium ion stimulated the Na⁺ uptake more effectively than K⁺. Replacing the potassium in the uptake medium with choline inhibited most of the Na⁺ uptake (Table III and Fig. 8). However, there was a small amount of Na⁺ uptake (<20%) in the absence of K⁺ as has already been described for the (Na⁺,K⁺)-pump (49). These observations are consistent with the known characteristics of the (Na⁺,K⁺)-pump (32, 48). Substituting 5 mM NaCl for 5 mM KCl in the assay medium did not change the kinetics of Na⁺ uptake and final capacity, confirming that mitochondrial ATPase was not involved.

Low concentrations of digitoxin (10 μM), a lipid-soluble cardiac glycoside (44), completely inhibited the sodium uptake (Fig. 8). Ouabain, an impermeant cardiac glycoside, had no effect on the Na⁺ uptake rate (Table III), consistent with the view that the inhibitory site of ouabain is on the interior of the vesicle.

Vanadate, at low concentrations, inhibited the Na⁺ uptake (Figs. 9 and 11). The concentration required for half-maximal inhibition was between 100 and 200 mM, similar to that reported by Karlsh and Pick (47) for reconstituted (Na⁺,K⁺)-ATPase isolated from pig kidney outer medulla. Vanadate has been shown to bind to the site of ATP hydrolysis of the (Na⁺,K⁺)-ATPase (45, 46) that is on the outer face of inside-out vesicles (Fig. 4).

Rubidium Efflux Studies—By pre-equilibrating the vesicles with 66Rb, which can substitute for K⁺, we confirmed that Na⁺ inward pumping was coupled to Rb⁺ efflux as expected for an operating (Na⁺,K⁺)-pump (50, 51). Not all of the 66Rb was pumped out of these vesicles as sodium was pumped in (Fig. 10), perhaps reflecting a higher permeability of the vesicle to Rb⁺ or K⁺ under these conditions as well as the presence of some right-side-out vesicles which do not pump ions under the conditions of the assay.

Toxin-activated Na⁺ Channels—The plasma membrane of electrically excitable tissues contains Na⁺ channels that increase membrane sodium permeability when depolarized or when incubated with certain plant or animal toxins (52). Batrachotoxin, at low concentrations, causes depolarization of excitable membranes by opening Na⁺ channels (53).

In order to investigate the presence of Na⁺ channels in our isolated plasma membrane vesicles, we incubated them with batrachotoxin (10 μM), which prevented most of the ATP-dependent Na⁺ accumulation by the plasma membrane vesicles (Fig. 11). Batrachotoxin (10 μM) had no effect on the ouabain-sensitive (Na⁺,K⁺)-ATPase activity measured after pretreatment with SDS, suggesting that batrachotoxin did not

![Graph](image-url)
cotoxin since nearly inhibit the Na+ pump; this is consistent with the observations of others (55). This experiment suggests that most of the vesicles had Na+ channels which were activated by batrachotoxin since nearly all the Na+ accumulation was prevented by batrachotoxin (Fig. 11). Veratridine (100 μM) also prevented Na+ accumulation (data not shown), but was not as effective as batrachotoxin (54).

**DISCUSSION**

A method is described for isolating sealed plasmalemma vesicles from rabbit skeletal muscle. The preparation is highly enriched in plasma membrane marker enzymes, containing the highest specific activity of ouabain-sensitive (Na+,K+)-ATPase, adenylate cyclase, and acetylcholinesterase activities (Table I) reported for a skeletal muscle plasmalemma preparation (Table IV). It is practically devoid of mitochondria and sarcoplasmic reticulum as indicated by low activities of diagnostic enzymes (Table I). The vesicles are largely impermeable to substrates and ions, making them especially suitable for ion transport studies. The sidedness has been characterized
purified preparation was practically eliminated with the use of plasma membrane marker enzymes (57). The leaky membranes (56) were used in the assay medium to reduce ATPase from the latter must be subtracted from the ouabain-sensitive (Na⁺,K⁺)-ATPase in order to estimate inside-out vesicles. To minimize the basal ATPase, EGTA and azide were used in the assay medium to reduce ATPase from sarcolemmal reticulum and mitochondria, respectively. Inactivation of basal ATPase by detergent treatment would result in an inflated estimate of the amount of inside-out vesicles. Therefore, the use of detergent to optimize activity must be carefully calibrated.

There is little experience in the use of acetylcholinesterase to estimate membrane sidedness in muscle. Most of the acetylcholinesterase activity from muscle is associated with the extracellular surface of the plasmalemma as determined by histochemistry (42), although a small portion is located intracellularly (64, 65). Some acetylcholinesterase appears to be associated with the basal lamina (66, 67); we have no indications that the latter isolates with the plasmalemma preparation described here. There is heterogeneity in the distribution of this enzyme in skeletal muscle sarcolemma. Approximately one-third is localized at the neuromuscular junction (64, 66, 68). Therefore, caution must be exercised since a small fraction of the vesicle population could skew the interpretation for the entire population. Pragmatically, the use of acetylcholinesterase to assess membrane sidedness is valid for this preparation. It gives similar results to the ouabain-dependent (Na⁺,K⁺) ATPase.

Two approaches were used to estimate membrane sidedness in our studies. Both approaches use the latency of ouabain-sensitive (Na⁺,K⁺)-ATPase for the quantitation of sealed vesicles. Sealed vesicles do not display this activity since both faces of the membrane must be accessible in order to measure this activity. The first procedure combines this assay with the latency of acetylcholinesterase to measure the amount of inside-out vesicles (Fig. 5 and Table II). The second procedure uses only the ouabain-sensitive (Na⁺,K⁺)-ATPase assay by itself. The percentage of right side-out vesicles is obtained as the difference in ATPase rate with and without detergent pretreatment (Fig. 6). Both procedures make use of SDS pretreatment to release the latency in order to express total activity.

Monsen in is an ionophore which, when added to the assay medium, dissipates the Na⁺ and K⁺ gradients generated by the (Na⁺,K⁺)-pump of the vesicles (Fig. 7). Therefore, initial rates of (Na⁺,K⁺)-ATPase in inside-out vesicles can be obtained. ATPase activity is enhanced because Na⁺, which would otherwise compete with K⁺ for binding sites on the inner face, does not concentrate. Also, K⁺, which is required on the inner face for optimal (Na⁺,K⁺)-ATPase activity, can leak in.

The measurement of ATPase activity includes "basal" ATPase, i.e., MgATPase which is not sensitive to ouabain (6). The latter must be subtracted from the ouabain-sensitive (Na⁺,K⁺)-ATPase to estimate inside-out vesicles. To minimize the basal ATPase, EGTA and azide were used in the assay medium to reduce ATPase from sarcolemmal reticulum and mitochondria, respectively. Inactivation of basal ATPase by detergent treatment would result in an inflated estimate of the amount of inside-out vesicles. Therefore, the use of detergent to optimize activity must be carefully calibrated.

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ATPase method which makes use of monensin. Furthermore, a third estimate of membrane sidedness used in our laboratory, the latency of (3H)ouabain binding sites, leads to the same conclusion that most of the vesicles are sealed and oriented inside out.

The plasma membrane preparation described in this report contains mostly inside-out plasma membrane vesicles and is especially suitable for Na⁺ transport studies energized by ATP. This is the first such study with skeletal muscle plasma membrane. Na⁺ gradients can be generated which are 10-fold, comparable with that in the muscle fiber. It is clear that the pumping is achieved by the (Na⁺,K⁺)-pump of the skeletal muscle plasma membrane since 1) it is energized by ATP and there is concomitant K⁺ efflux with Na⁺ uptake; 2) the characteristics of non-Na⁺ ions required for activation are similar to that reported for the (Na⁺,K⁺)-pump from other systems; 3) the pumping is inhibited by cardiac glycosides and vanadate comparable with the (Na⁺,K⁺)-pumps which have been studied; and 4) batrachotoxin and veratridine obviate Na⁺ accumulation, indicating that an excitabile membrane containing Na⁺ channels is involved.

Lau and co-workers have isolated a transverse tubule preparation from skeletal muscle which is mainly inside-out and is capable of energized Na⁺ uptake. The plasma membrane vesicle preparation described in this report is mainly inside-out in orientation, but the indications are that it derives mainly from the surface and not from a transverse tubule. A transverse tubule has a morphologically distinctive appearance, i.e. flattened tubules, 0.1 × 0.04 μm, with electron opaque “caps” at opposite ends. Thin section electron microscopy of the plasmalemma preparation reveals more rounded although irregular shaped vesicles averaging 0.2-0.5 μm in diameter. It did not reveal appreciable transverse tubules (Fig. 2) although the latter were present in other fractions from the sucrose gradient (Fig. 1A, Band 3).

The plasma membrane preparation (PMd) described here has several-fold higher (Na⁺,K⁺)-ATPase activity than has been obtained for isolated transverse tubules (Table IV). This is consistent with ouabain binding studies on intact tissue in which a much higher density of (3H)ouabain binding sites was measured on the plasmalemma than in the transverse tubule (62). It is interesting to note that the Ca²⁺-ATPase activity in our plasma membrane-enriched fraction is very low compared with that reported for transverse tubule (60, 61, 63).

The time required to reach maximal Na⁺ uptake capacity for these vesicles occurs within 12 min and is more than 5-fold faster than the transverse tubules isolated from triads (29). The Na⁺ flux is faster in PMd than transverse tubule, possibly reflecting the larger amount of (Na⁺,K⁺)-ATPase in PMd as compared with the transverse tubule preparation (Table IV).

Progress in the study of heart plasmalemma sidedness and transport, thus far, has outpaced studies with skeletal muscle (30, 70, 71, 75).

The best characterized cardiac sarcolemma preparation by Jones et al. (22) is largely right side-out. This preparation does not concentrate Na⁺ and would not be expected to since it is predominantly right side-out. Nonetheless, it has a 2-fold higher specific activity of ouabain-sensitive (Na⁺,K⁺)-ATPase and adenylate cyclase than does our skeletal muscle preparation. This difference between skeletal and cardiac muscle plasma membranes probably reflects different characteristics of the two types of muscle tissue.

Heart plasmalemma preparations have been reported which are capable of concentrating Na⁺ (31, 69). Grosse et al. (31) have prepared cardiac vesicles which appear to be largely inside-out and these are capable of pumping Na⁺ with similar specificity to skeletal muscle PMd, although the rate of Na⁺ pumping and the concentration gradient which is generated are not given. An interesting aspect of their study is the interaction of the (Na⁺,K⁺)-ATPase with creatine phosphokinase. Creatine phosphokinase was found associated with their cardiac plasma membrane preparation (30), and an added ATP-regenerating system stimulated both the Na⁺ uptake and (Na⁺,K⁺)-ATPase. We also found that an ATP-regenerating system stimulated Na⁺ uptake.

There are major difficulties in the isolation of a defined muscle plasma membrane from skeletal muscle. The muscle fibers are entrapped in an extensive collagen network, making them resistant to disruption. The plasma membrane is heterogeneous, and the surface membrane represents only a small portion of the plasma membrane which invaginates, giving rise to transverse tubule (71). In frog skeletal muscle, Peachey (72) has estimated that the outer 6 times the transverse tubule than surface membrane, and there is 5-6 times as much sarcoplasmic reticulum as transverse tubule. Only recently have transverse tubule preparations been described (60, 61, 63), and their diagnostic characteristics are not known with any degree of certainty. There is no marker enzyme available to discriminate between transverse tubule and surface membrane. The characterization of membrane sidedness is not trivial. The aspect of membrane sidedness is a dimension which has not previously been considered for skeletal muscle plasma membrane isolation. Another problem is that contractile filaments, which comprise most of the muscle mass, serve to “glue” the components together, making membrane separation more difficult and the yield low.

Early skeletal muscle plasma membrane isolation techniques yielded a “sarcolemma” or outer sheath preparation. The sarcolemma surrounds the muscle fiber (4, 5, 72) and consists of three distinct layers, collagen, glycocalyx, or basal lamina and plasma membrane (70). The sarcolemma preparation was prepared from muscle by homogenization and subsequent extraction with 0.4 M LiBr and later with 1.0 M KCl which solubilizes the contractile proteins and releases internal organelles (4). Another sarcolemma preparation utilized homogenization in Ca²⁺-containing solutions and incubation at 37 °C, at which temperature the contractile proteins become more soluble and endogenous proteolysis likely takes place. Plasma membranes can be released from sarcolemma preparations by additional shear, and purification can be achieved with the use of sucrose density gradient centrifugation (8, 9, 11, 73). Yet another approach to plasma membrane isolation utilized direct and vigorous homogenization of the muscle tissue to obtain microsomes. The microsomes were then fractionated on a sucrose density gradient (15, 16).

The isolation of highly purified plasma membrane vesicles from rabbit skeletal muscle described here makes use of a limited blending in high salt, followed by homogenization in buffered sucrose. Two gradients are used sequentially for purification, including a second dextran gradient which removes leaky vesicles. The preparation appears to consist mainly of surface membrane rather than transverse tubule. The surface membrane fraction is largely sealed and oriented inside out. The vesicles have been shown to effectively generate a sodium ion gradient, the magnitude of which is comparable with that in the muscle fiber.

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1. P. Volpe, S. Seiler, and S. Fleischer, unpublished data.
2. R. Mitchell, A. Saito, P. Palade, and S. Fleischer, manuscript submitted for publication.
3. A. Saito, S. Seiler, and S. Fleischer, manuscript in preparation.
