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IN VITRO STUDIES OF SKELETAL MUSCLE MEMBRANES

CHARACTERIZATION OF A PHOSPHORYLATED INTERMEDIATE OF SARCOLEMMAL $(Na^+ + K^+)$ATPase*

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SUMMARY

The sarcolemmal membranes isolated from rat skeletal muscle are capable of incorporating ^{32}P from [γ-^{32}P]ATP. The membrane protein phosphorylation requires Mg^{2+}. Cyclic AMP, cyclic GMP and their dibutyryl derivatives showed no marked effect on sarcolemmal phosphorylation.

The Mg^{2+}-dependent ^{32}P labeling was significantly enhanced by Na^+. The rate of Na^+-stimulated ^{32}P incorporation was quite rapid reaching steady state levels within 5 s at 0 °C. K^+ reduced the Na^+-stimulated ^{32}P-incorporation but enhanced the $^{32}P_i$ release. This inhibitory effect of K^+ on Na^+-stimulated ^{32}P incorporation was prevented by the cardiac glycoside, ouabain.

The Na^+-dependent ^{32}P labeling showed substrate dependency and the Na^+ site was saturable. The apparent K_m for ATP was $2 \cdot 10^{-5}$ M. The optimum pH for ^{32}P labeling was between 7 and 8.

Na^+-dependent membrane phosphorylation showed a direct relationship with the $(Na^+ + K^+)$ATPase activity. The high turnover rate of ^{32}P intermediate (12 000 min^{-1}) suggested its functional significance in the overall transport ATPase reaction sequence.

The predominate portion (> 90 %) of the phosphorylated membrane complex was sensitive to acidified hydroxylamine and to alkaline pH suggesting an acylphosphate nature of the phosphoprotein.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed that ^{32}P incorporation occurred predominately into a 108 000 dalton subunit which is a major

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Abbreviations: $(Na^+ + K^+)$ATPase, Na^+ plus K^+-stimulated, Mg^{2+}-dependent adenosine triphosphatase (EC 3.6.1.3); cyclic AMP, cyclic adenosine 3′ : 5′-monophosphate; cyclic GMP, cyclic guanosine 3′ : 5′-monophosphate.

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protein component of sarcolemmal membranes. A very low level of ^{32}P incorporation was also observed into a 25 000 dalton subunit and Ca^{2+} slightly enhanced the phosphorylation of this component.

The size (M_r 108 000) and some properties of the sarcolemmal phosphoprotein are closely similar to other ($Na^+ + K^+$)ATPase preparations reported so far.

INTRODUCTION

The unique biological properties of excitable cell membranes are related to their special ability to critically control the selective permeability to ions such as Na^+ and K^+ and probably Ca^{2+} [1–4]. Electrochemical gradients for Na^+ and K^+ are known to be maintained across the muscle fiber membrane, as they are in many other cells, primarily through the action of a sodium pump (see ref. 5 for details). However, controversy exists as to the relative importance of exchange diffusion and active transport in maintaining the sodium pump in skeletal muscle [5–8].

A growing body of evidence has accumulated to date suggesting that active transport of sodium out and potassium into the cell is enzymatically expressed as the membrane-bound ($Na^+ + K^+$)ATPase [9–10]. A membrane protein transiently phosphorylated under the influence of Na^+ has been described in many of those microsomal preparations that are enriched with ($Na^+ + K^+$)ATPase activity. This phosphoprotein component has been identified as an intermediate in the reaction sequence catalyzed by ($Na^+ + K^+$)ATPase. Certain characteristics of the phosphoprotein intermediate have led to the suggestion of its participation in the reaction mechanisms whereby the phosphate bond energy may be utilized to do the useful transport work (see refs. 9 and 10 for recent reviews of the subject).

The molecular components of skekelal muscle underlying monovalent cation transport have not yet been fully defined. Because the phosphorylation of membrane proteins appears to have biological significance and because such information is not known in detail for skeletal muscle plasma membrane, we have investigated some properties of phosphorylation of an isolated sarcolemmal fraction. These sarcolemmal particles have been previously [11] characterized and shown to contain many surface membrane functions. Therefore, these particles afford a relatively simple and useful in vitro model to explore the membrane biology of an excitable tissue. The present study demonstrates that the phosphorylation of sarcolemmal membranes is profoundly influenced by monovalent cations, Na^+ and K^+, while cyclic nucleotides have no marked effect. Gel electrophoretic analysis and the chemical properties of the phosphoprotein indicate that the majority of the ^{32}P incorporation represents the phosphorylation of membrane bound transport ATPase.

MATERIALS AND METHODS

Materials

Male Sprague-Dawley rats were supplied by Zivic-Miller Labs (Allison Park, Pa). Radioactive compounds, [γ-^{32}P]ATP (> 10 Ci/mmol) and [α-^{32}P]ATP (5–10 Ci/mmol) were obtained from New England Nuclear (Boston, Mass.). Sucrose,

ultrapure grade, was purchased from Schwarz-Mann (Orangeburg, N.Y.). ATP, cyclic AMP, cyclic GMP and their dibutyryl derivatives were obtained from Sigma Chemical Company (St. Louis, Mo.). Reagents for acrylamide gel electrophoresis were supplied by Eastman Company (Rochester, N.Y.). Sodium dodecyl sulfate was purchased from British Drug House (Poole, England). Dithiothreitol was from Calbiochem (La Jolla, Calif.). The reference proteins were obtained either from Sigma or Worthington Biochemical Corporation (Freehold, N.J.) All other reagents were of analytical grade.

Isolation of sarcolemmal membranes

The sarcolemmal membranes were isolated from the gastrocnemiusplantaris muscles as previously described [11] except that the initial homogenization was carried out with a Polytron PT 10 homogenizer (Brinkman Instruments, Westbury, N.Y.) for a total of 90 s; for 1 min at a setting of 8 and at 5 for an additional 30 s. All the operations were carried out at 2–4 °C. The homogenate was centrifuged at $1000 \times g$ for 10 min. From the pellet, a particulate fraction was obtained by differential centrifugation after sequential LiBr and KCl extraction [12]. The membrane fraction was finally washed twice with deionized H_2O containing 0.2 mM H_4EDTA neutralized with Tris base. The washed membranes were suspended in the same medium and used in the present experiments. Further purification of the membranes was achieved by discontinuous sucrose density gradient separation. Discontinuous gradients of 0.4–1.4 M sucrose (10 mM Tris · Cl, 0.2 mM H_4EDTA, pH 8.4) in 0.2-M steps were prepared in 11-ml cellulose nitrate ultracentrifuge tubes. The sarcolemmal membranes (15–20 mg/ml) in aliquots of 0.9 ml were layered on top of the gradient. The tubes were centrifuged in a Beckman SW 41 Ti rotor at $200\,000 \times g_{max}$ for 3.5 h in a Beckman L5-65 ultra-centrifuge. The membrane fractions settled at the interphases of 0.6–0.8 M (SLF_1), 0.8–1.0 M (SLF_2), 1.0–1.2 M (SLF_3), 1.2–1.4 M (SLF_4) and the pellet (SLF_5) were recovered and washed with excess solution of 10 mM Tris, 0.2 mM H_4EDTA, pH 7.4 to remove sucrose and pelleted by centrifugation at $200\,000 \times g$ for 1 h in a 60 Ti fixed-angle rotor. The pellets were resuspended in the same medium and used for the assays.

The density gradient profile obtained in the present study slightly differs from that reported earlier, where 5 distinct bands at the interphases and a pellet were obtained [11]. It is our present experience that the band at 0.4–0.6 M sucrose interphase noted in the previous study is no longer as distinct when initial homogenization was carried out with a Polytron PT 10 homogenizer. Thus, the SLF_1 fraction in the present study corresponds to our previously reported SLF_1, and SLF_2 fractions. The biochemical properties of these fractions reported earlier [11] were recovered in the fraction designated SLF_1 in the present study.

Phosphorylation of sarcolemmal membranes

The standard reaction mixture, in a total volume of 1 ml at pH 7.5, contained 0.2–0.3 mg membrane protein, 25 mM Tris, 0.1 mM [γ-^{32}P]ATP ($1–3 \cdot 10^7$ dpm/tube) other ions and cyclic nucleotides were present at indicated concentrations in the appropriate experiments. The membranes and reaction mixture lacking [γ-^{32}P]ATP were allowed to equilibrate for 15–20 min at 0 °C. The reaction was initiated by the addition of radioactive ATP. The mixture was then incubated at 0 °C for 10 s unless

otherwise indicated. The reaction was terminated by rapidly injecting 5 volumes of an ice-cold "stopping solution" containing 6 % (w/v) trichloroacetic acid, 50 mM KH_2PO_4 and 1 mM non-radioactive ATP. Control tubes were always carried out in parallel when either boiled membranes were used or the "stopping solution" was added prior to the addition of radioactive ATP. The contents were centrifuged at $3000 \times g$ for 15 min at 4 °C. The pellet was washed 3 times with "stopping solution" and once with ice-cold, glass-distilled H_2O. The supernatant of the final wash was essentially free of any radioactivity. The washed pellet was dissolved in a solution containing 2 % sodium dodecyl sulfate, 4 mM dithiothreitol and 50 mM Tris/HCl, pH 7.4. The tubes were incubated for 20–30 min at 60 °C in a waterbath. Suitable aliquots were counted with 10 ml Aquasol (New England Nuclear) either in a Packard Tricarb Model 3375 or in a Beckman LS 355 at 98 % efficiency. The values are expressed unless otherwise noted as pmol ^{32}P bound per mg protein per 10 s assay.

In the experiments where electrophoresis was also performed the concentration of [γ-^{32}P]ATP in the reaction medium was reduced to 0.01 mM ($1–3 \cdot 10^7$ dpm/tube) in order to obtain more radioactivity in the labeled proteins. Neither the binding pattern nor the electrophoretic profile of sarcolemmal membranes were altered by the decreased ATP concentration. The phosphorylation and subsequent washings were carried out essentially as described above. The washed pellets were directly dissolved in sample solution used to solubilize the proteins in the electrophoresis procedure described below.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

The disc gel electrophoresis procedure closely followed the method of Fairbanks et al. [13]. 5.6 % acrylamide gels were used to analyse the electrophoretic pattern of phosphorylated membranes. The protein samples were dissolved in a medium containing (final concentrations): 1 % sodium dodecyl sulfate, 40 mM dithiothreitol, 0.01 % Pyronin Y (tracking dye), 5–10 % sucrose and 10 mM Tris, pH 8.0. The samples were incubated for 20 min at 45 °C in a water bath. These conditions ensured complete solubilization of protein samples as very little or no aggregation of protein (judged by Coomassie blue staining) nor appreciable radioactivity stayed at the top of the gel. Higher temperatures caused extensive loss of radioactivity. After solubilization, 40-μl portions (60–80 μg protein) were subjected to electrophoresis in duplicate. The gels were run at 8 mA per gel in 1 % sodium dodecyl sulfate, 40 mM Tris/acetate, pH 7.4 in a Bio-Rad apparatus (Bio-Rad Laboratories, Richmond, Calif.). Tap water was circulated through the electrophoresis chamber at a temperature of 16 °C. When the tracking dye moved 8 cm, the gels were removed from their tubes and one of each duplicate pair was immediately frozen on solid CO_2 and cut transversely into approximately 1 mm sections with a wire cutter device. The gel discs were transferred into counting vials and treated overnight at 50 °C with 0.5 ml 30 % H_2O_2. After cooling the samples to room temperature, 10 ml of Aquasol were added to the vials and counted.

The second gel was fixed, stained, and destained essentially as described [13]. The stained gels were photographed and scanned at 520 nm with a Gilford Model 2410 with a linear transport attachment. After scanning, the gels were sliced longitudinally [14] and the gel slices were layered onto a wet Whatman No. 1 filter paper and dried under vacuum. The gels were placed in contact with Kodak NS-54 medical

X-ray film generally for 4–7 days and the film was developed. The resulting autoradiograph revealed those proteins into which radioactive phosphate had been incorporated during incubation.

Gels were calibrated with five proteins of known molecular weight: β-galactosidase (130 000; Worthington), phosphorylase *a* (94 000, Sigma), bovine serum albumin (68 000, Pentex,) aldolase (40 000, Sigma) and ribonuclease (13 700, Sigma). The standard protein mix was always run alongside the samples.

Hydroxylamine and pH treatment of phosphorylated membranes

In some experiments, trichloroacetic acid-precipitated and washed ^{32}P-labeled sarcolemmal membranes were resuspended in distilled H_2O and treated either with solutions of different pH or with different concentrations of freshly prepared hydroxylamine in 0.1 M acetate buffer (pH 5.4) for 30 min at room temperature. For the control, hydroxylamine was replaced with KCl (pH 5.4). The reaction was stopped by adding an ice-cold solution of 20 % trichloroacetic acid, and centrifuged. The radioactivity in both the supernatant and pellet (after solubilization with sodium dodecyl sulfate) was determined by liquid scintillation spectrometry, and the percentage of ^{32}P released was calculated.

ATPase assay

The standard reaction medium contained appropriate amounts of sarcolemmal membranes, 3 mM Tris/ATP, 7.5 mM $MgCl_2$, 100 mM NaCl, 20 mM KCl, 0.75 mM EDTA, 30 mM imidazole/glycylglycine, pH 7.5: when present, ouabain was at 0.1 mM. The reaction was for 10 min at 37 °C. The liberated phosphate was determined by the method of Post and Sen [15]. Butanol extraction procedure [16] was employed to estimate the radioactive inorganic phosphate shown in Fig. 1.

Protein was determined by the method of Lowry et al. [17] using bovine serum albumin as a standard.

RESULTS

The isolated sarcolemmal membranes are very active in accumulating ^{32}P from [γ-^{32}P]ATP into a trichloroacetic acid-pricipitatable residue. This phosphorylation required Mg^{2+} (optimal at 1 mM). Denaturation of membranes by exposure to trichloroacetic acid or heat inactivation (100 °C for 5 min) completely abolished the ^{32}P incorporation. The membrane phosphorylation was specific for terminally-labeled ATP as no significant phosphorylation occured if alpha-labeled ATP was used as substrate. Preliminary results (not shown) also indicated that the sarcolemmal protein phosphorylation was not influenced by cyclic AMP or cyclic GMP nor by their dibutyryl derivatives.

Effects of different ions and ouabain on sarcolemmal phosphorylation

The sarcolemmal phosphorylation was profoundly influenced by inorganic ions. Of the several monovalent cations tested (Li^+, K^+, Na^+, NH_4^+, Ru^+, Cs^+), only Na^+ enhanced ^{32}P incorporation by three to four fold. When K^+ was also present along with Na^+, the incorporation was reduced to that observed in the presence of Mg^{2+} alone (Table I). Thus, Na^+ stimulates phosphorylation while K^+

TABLE I

EFFECT OF DIFFERENT IONS AND OUABAIN ON SARCOLEMMAL PHOSPHORYLATION AND ATPase ACTIVITIES

Phosphorylation and ATPase activity of sarcolemmal membranes were carried out as described in Methods. When present the concentrations of Mg^{2+}, Na^+, K^+, Ca^{2+} and ouabain were 1, 100, 20, 1 and 0.1 mM respectively. Values are means of 6 observations ±S.E.

Ions and/or ouabain	Phosphorylation (pmol/mg)	ATPase activity (μmol P_i/mg/h)
nil*	0.5±0.2	8.6±0.5
Mg^{2+}	15.0±2.0	11.5±1.3
$Mg^{2+}+Na^+$	46.0±3.2	10.8±2.0
$Mg^{2+}+K^+$	14.0±2.6	11.0±2.5
$Mg^{2+}+Na^++K^+$	19.0±1.8	29.0±2.6
Mg^{2+}+ouabain	15.5±3.8	10.0±2.0
$Mg^{2+}+Na^+$+ouabain	39.3±2.5	10.5±1.5
$Mg^{2+}+Na^++K^+$+ouabain	35.6±4.2	9.8±1.4
$Mg^{2+}+Ca^{2+}$	11.5±2.0	12.2±0.8
Ca^{2+}	8.6±1.8	9.4±1.2

* Contained 1 mM EDTA.

abolishes the Na^+ stimulation. These observations are consistent with the known properties of ^{32}P intermediate of (Na^++K^+)ATPase [9, 10]. Results of Table I also indicate the relationship between levels of phosphoprotein and hydrolytic activity of sarcolemma in the presence of different ions and ouabain, a known inhibitor of (Na^++K^+)ATPase. Higher ATPase activity and lower phosphoprotein in the presence of Na^+ and K^+ suggests that K^+ enhances dephosphorylation and thereby promotes phosphate release. Ouabain did not markedly affect the Na^+-dependent phosphorylation while it prevented the K^+-stimulated dephosphorylation and thereby inhibited the (Na^++K^+)ATPase.

Kinetic properties of Na^+-stimulated phosphorylation reaction

Time course of ^{32}P incorporation is shown in Fig. 1. As can be seen the Na^+-dependent phosphorylation reached a steady state level within 5 s and remained constant for at least 60 s. However, if K^+ was also present in the reaction medium simultaneously with Na^+, the extent of labeling was 3–4 fold lower at all periods of incubation investigated (Fig. 1). Estimation of inorganic phosphate under the same conditions in the trichloroacetic acid supernatants revealed that more phosphate was released in the presence of K^+ than in its absence (Fig. 1).

Dependence of membrane protein phosphorylation on Na^+ concentration is shown in Fig. 2. The amount of phosphoprotein increased steadily with increasing Na^+ concentration. The rate increased at low concentrations and then plateaued at higher concentrations. The concentration of Na^+ required for half-maximal effect was approx. 1 mM. In contrast, K^+ at all concentrations tested (Fig. 2) did not alter the basal ^{32}P incorporation noted in the presence of Mg^{2+} alone. These studies clearly indicate the specific ion requirements for phosphorylation reaction in that only Na^+ enhances the ^{32}P labeling while K^+ inhibits the Na^+ stimulation (Fig. 1).

Na^+-stimulated, membrane-protein labeling showed a strong dependency on ATP concentration (Fig. 3). The apparent K_m for ATP was $2 \cdot 10^{-5}$ M, a value slightly higher than that reported for kidney [18] and brain [19] ($Na^+ + K^+$)ATPase preparations.

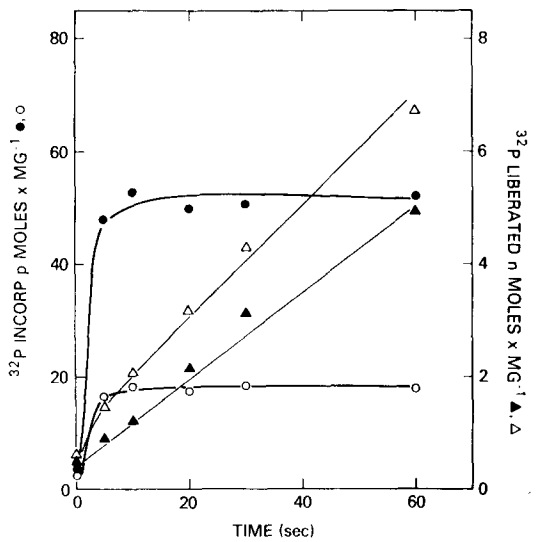

Fig. 1. Time course of ^{32}P incorporation (○, ●) and $^{32}P_i$ release (△, ▲) by sarcolemmal membranes in the presence of 100 mM Na^+ (●, ▲) or 100 mM Na^+ +20 mM K^+ (○, △). The other conditions were 1 mM Mg^{2+}, 0.1 mM [γ-^{32}P]ATP and 25 mM Tris/HCl, pH 7.5. The reaction was carried out at 0 °C.

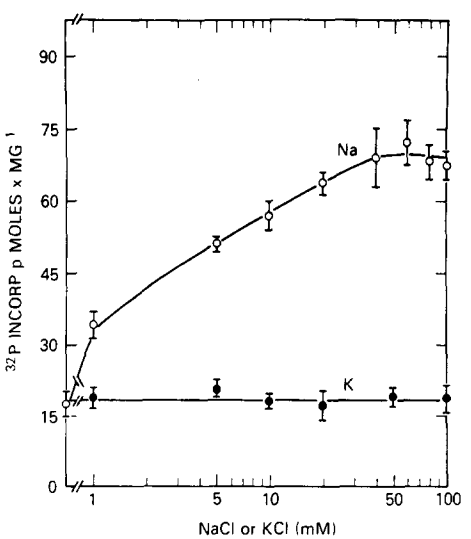

Fig. 2. Effects of Na^+ and K^+ on the Mg^{2+} dependent phosphorylation of sarcolemmal membranes. The reaction medium contained 0.1 mM [γ-^{32}P]ATP, 1 mM Mg^{2+}, 25 mM Tris/HCl, pH 7.5 and the concentrations of Na^+ or K^+ are as shown.

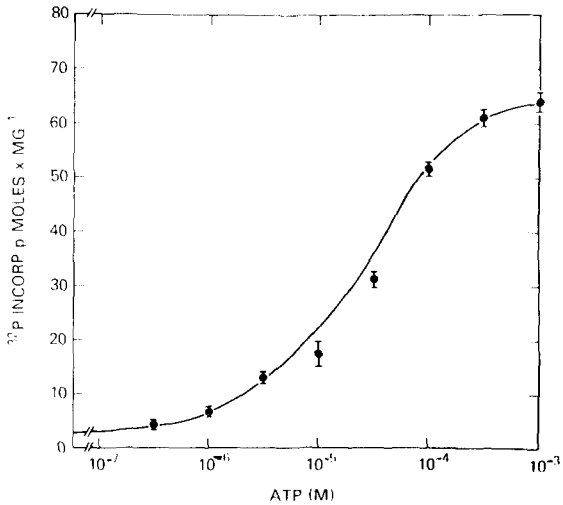

Fig. 3. Dependency of Na^+-stimulated ^{32}P labelling on ATP concentration. The reaction conditions were essentially similar as described in Methods, except the ATP concentration was varied as indicated. Values are means of 4 observations.

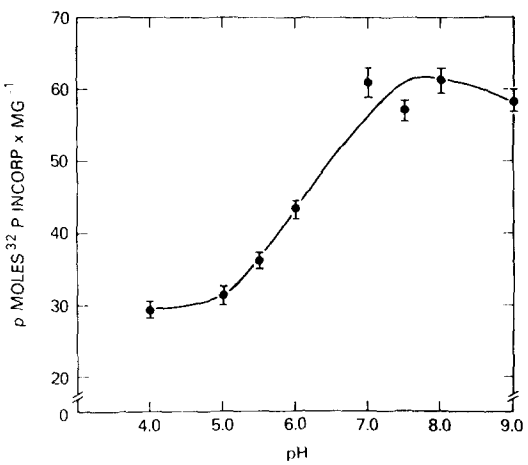

Fig. 4. pH dependency of Na^+-stimulated sarcolemmal phosphorylation. The reaction medium contained 0.1 mM [γ-^{32}P]ATP, 1 mM Mg^{2+}, 100 mM Na^+ and 50 mM Tris/maleate adjusted to the required pH. Values are mean of 4 observations.

The phosphorylation of membranes did not display a sharp dependency on pH (Fig. 4). The ^{32}P incorporation was readily measurable from pH. 4.0 to pH 9.0. However, the maximum labeling occurred between pH 7.0 and pH 8.0.

Effect of ADP on ^{32}P incorporation

ADP, an end product of the $(Na^+ + K^+)$ATPase reaction strongly inhibited the Na^+-dependent phosphorylation of sarcolemmal membranes. Fig. 5 shows the results when ATP was kept at a fixed concentration (0.1 mM) and ADP concentration

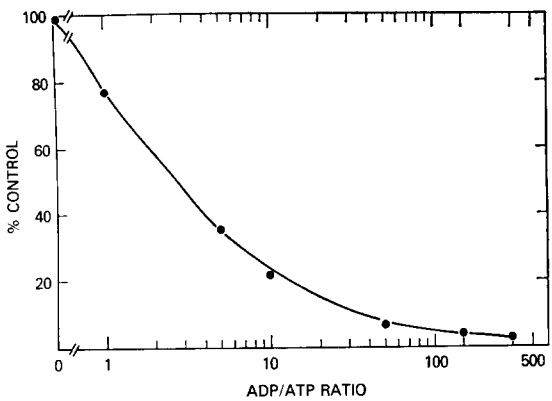

Fig. 5. Effect of ADP on Na^+-stimulated ^{32}P labeling of sarcolemmal membranes. The reaction mixture contained 0.1 mM [γ-^{32}P]ATP, 1 mM Mg^{2+}, 100 mM Na^+, 25 mM Tris/HCl, pH 7.5 and varying amounts of ADP.

was varied to obtain different ADP/ATP ratios. At a ratio of 5 the ^{32}P incorporation reduced to 25 % of the control value. A ratio of above 50 completely abolished the membrane labeling. Higher levels of ADP thus seems to interfere with the normal phosphoprotein reaction sequence of $(Na^+ + K^+)$ATPase and thereby inhibits its hydrolytic activity. This suggests the features of end-product inhibition and correlates with the conclusion that the predominant phosphorylation activity of sarcolemmal membranes involves the ATPase intermediate.

Relationship between Na^+-dependent phosphoprotein and $(Na^+ + K^+)$ATPase

To evaluate further the association of Na^+-stimulated ^{32}P labeling to membrane-bound $(Na^+ + K^+)$ATPase, we directly compared these two activities. A plot

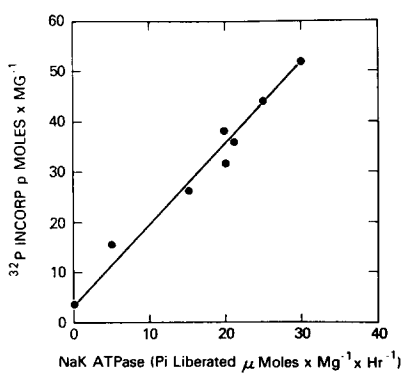

Fig. 6. Relationship between Na^+-stimulated ^{32}P labeling and $(Na^+ + K^+)$-stimulated ATPase activity of different sarcolemmal membrane preparations. The phosphorylation was carried out as described in the Methods. The ATPase assay conditions were 7 mM Mg^{2+}, 0.7 mM EDTA, 100 mM Na^+, 20 mM K^+ and 30 mM imidazole/glycylglycine, pH 7.5. The reaction was for 10 min at 37 °C. The activity obtained in a similar medium containing 0.1 mM ouabain was substracted and represented as $(Na^+ + K^+)$ATPase activity values.

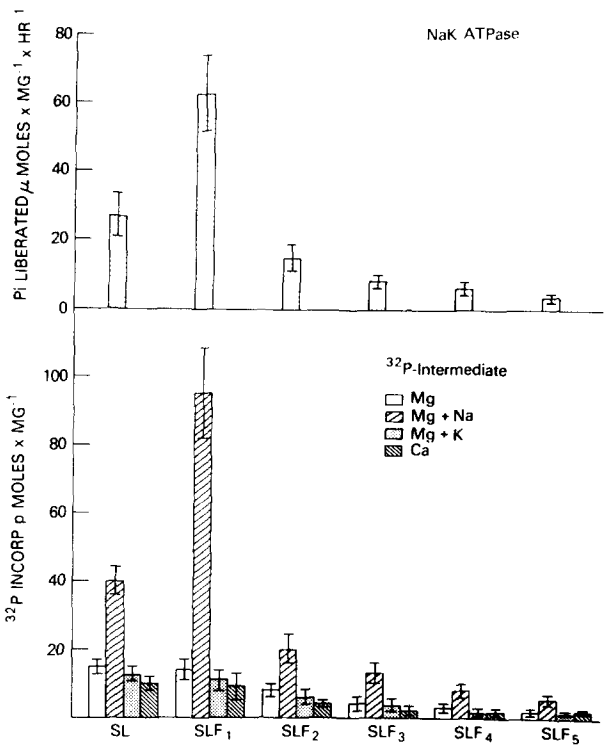

Fig. 7. The levels of $(Na^+ + K^+)$ATPase activity and the ^{32}P intermediate formed, under different ionic conditions as indicated, in sarcolemma and its subfractions obtained by sucrose density separation as described in Methods. $(Na^+ + K^+)$ATPase activity was estimated as described in Fig. 6. The phosphorylation reaction was carried out with 0.1 mM [γ-^{32}P]ATP, 25 mM Tris/HCl, pH 7.5 and when present Mg^{2+} 1 mM, Na^+ 100 mM, K^+ 20 mM and Ca^{2+} 1 mM. The values are mean of 5 different preparations.

of the amount of ^{32}P intermediate versus the hydrolytic activity for different preparations strikingly shows a direct relationship (Fig. 6).

A similar relationship was also seen in the further purified sarcolemmal subfractions obtained by sucrose density separation. Fig. 7 shows the distribution of ^{32}P intermediate and the $(Na^+ + K^+)$ATPase activity in different subfractions of crude sarcolemma. Of all the fractions only the SLF_1 fraction showed marked enrichment of both the $(Na^+ + K^+)$ATPase activity and ^{32}P labeling suggesting co-purification of these properties. Comparison of sarcolemmal and SLF_1 fractions revealed that while basal (Mg^{2+} alone) ^{32}P labeling remained unchanged, the Na^+-stimulated incorporation is significantly increased in the latter fraction. In the presence of Mg^{2+} and Na^+ the ^{32}P incorporation into SLF_1 was 8–10 times greater than that in the presence of Mg^{2+} alone; under similar conditions, crude sarcolemma showed only 3–4-fold stimulation by Na^+. Accordingly, the $(Na^+ + K^+)$ATPase activity of SLF_1 fraction was 40–65 μmol $\cdot$ mg^{-1} $\cdot$ h^{-1} compared to 20–30 μmol $\cdot$ mg^{-1} $\cdot$ h^{-1} obtained with the crude sarcolemmal fraction.

If the Na^+-dependent phosphorylated intermediate is a functional unit in ATP hydrolysis, the intermediate should undergo a rapid turnover in conjunction with ATP splitting. Thus, from the amount of ^{32}P intermediate and the ATPase activity a turnover number can be derived by making an assumption that one phosphate group in the intermediate is associated with one molecular unit of the enzyme and that all the ATP hydrolysis proceeds through the intermediate. The number thus obtained is the ratio of the number of phosphate groups liberated from ATP per min to the number of phosphate groups in the intermediate. A turnover number of 12 000 and 11 000 min^{-1} can be calculated for sarcolemmal and SLF_1 fractions respectively from the data in Fig. 7. The high turnover number supports the view that the phosphorylated protein is a functional intermediate in the reaction sequence catalyzed by $(Na^+ + K^+)$ATPase.

Nature of the phosphorylated intermediate

It is now well documented that at least two conformational forms of intermediates (E_1-P and E_2-P) are generated during the catalytic sequence of $(Na^+ + K^+)$ ATPase reaction [9, 10]. The two types of intermediates could be differentiated by their reactivity to K^+ and ADP. It has been shown that E_1-P form readily reacts with ADP but not with K^+ while the E_2-P intermediate possesses converse properties [9, 10]. Results shown in Fig. 8 suggest that the phosphoprotein of sarcolemma formed under standard assay conditions (0.1 mM ATP, 1 mM Mg^{2+} and 100 mM Na^+) dephosphorylated more readily by K^+ than by ADP and thereby indicate the E_2-P nature of the intermediate.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of phosphorylated membranes

Electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate was used to study the distribution of ^{32}P activity as well as to estimate the molecular weight of phosphoproteins of sarcolemmal membranes. We used the SLF_1 fraction

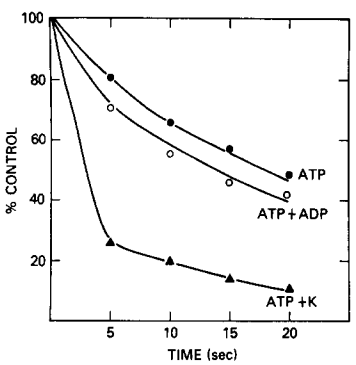

Fig. 8. Reaction of ^{32}P intermediate with ADP and K^+. 10 s after the additions of [γ-^{32}P]ATP to phosphorylate the membranes, the following additions were made at zero time and the reaction was stopped at the time points indicated. ●–● 2 mM unlabeled ATP; ○–○ 2 mM unlabeled ATP+1 mM ADP; ▲–▲ 2 mM unlabeled ATP+1 mM K^+. The values are plotted as a percentage of the amount of labeling at 10 s, i.e., prior to the various additions.

for some of these studies as it represented a more pure membrane preparation in terms of $(Na^+ + K^+)$ATPase activity and ^{32}P incorporation (Fig. 7). The results shown in Fig. 9 indicate the distribution of radioactivity along the length of a gel. As can be seen, most of the radioactivity migrated as a single peak corresponding to a major

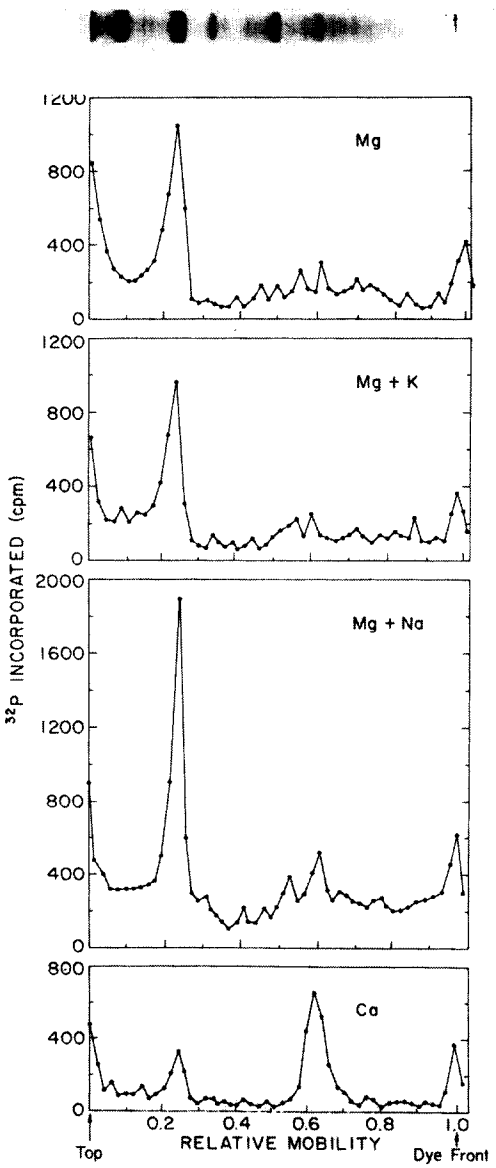

Fig. 9. Identification of phosphorylated sarcolemmal protein components on 5.6% polyacrylamide gels containing sodium dodecyl sulfate. Phosphorylation, subsequent electrophoresis of sarcolemmal membranes and analysis of radioactivity in the transversely sliced gels were as described in methods. The ionic conditions of phosphorylation reactions were Mg^{2+} 1 mM, Na^+ 100 mM, K^+ 20 mM and Ca^{2+} 1 mM.

protein band of sarcolemmal membranes. A minor, but variable, peak was also found to be associated with smaller proteins. The radioactivity into the larger component is enhanced in the presence of Na^+ and it remained at the Mg^{2+} level in the presence of K^+. Ca^{2+} reduced the ^{32}P incorporation considerably into the larger component but slightly enhanced the incorporation into the smaller component (Fig. 9). The Ca^{2+} effects are interesting but remain to be elucidated.

The above results were further confirmed by autoradiography. The auto-radiographs (Fig. 10) revealed that only a single radioactive band could be detected and on comparison with the protein stained gel indicates that the radioactive band comigrates with a major protein component. As observed earlier (Fig. 9) the autoradiograph also confirms that grain density is higher in the presence of Na^+ when compared to Mg^{2+} or K^+. Cyclic AMP or cyclic GMP were found to be without effect.

In earlier experiments the molecular weights of phosphoproteins were obtained

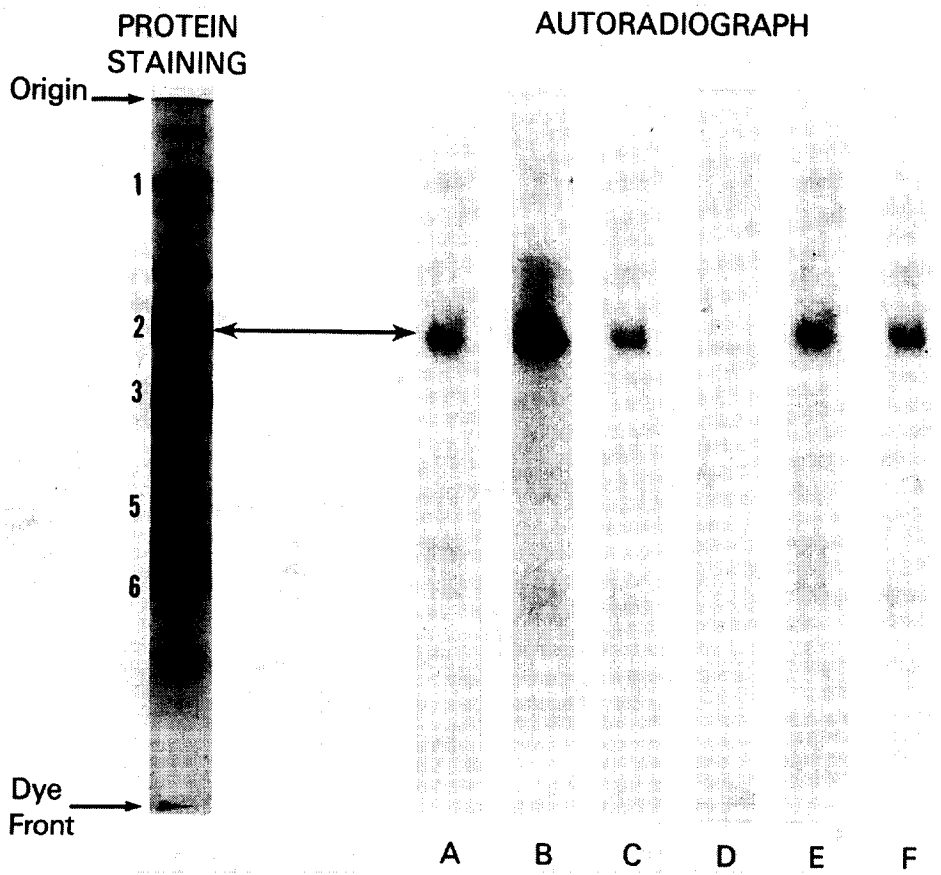

Fig. 10. Autoradiographic detection of phosphorylated SLF proteins under different conditions after sodium dodecyl sulfate polyacrylamide gel electrophoresis. A = 1 mM Mg^{2+}; B = 1 mM Mg^{2+} + 100 mM Na^+; C = 1 mM Mg^{2+} + 20 mM K^+, D = 1 mM Ca^{2+}, E = 1 mM Mg^{2+} + 0.01 mM cyclic AMP and F = 1 mM Mg^{2+} + 0.01 mM cyclic GMP. Typical protein pattern obtained from a duplicate gel is shown on the left which was essentially similar under different conditions of phosphorylation.

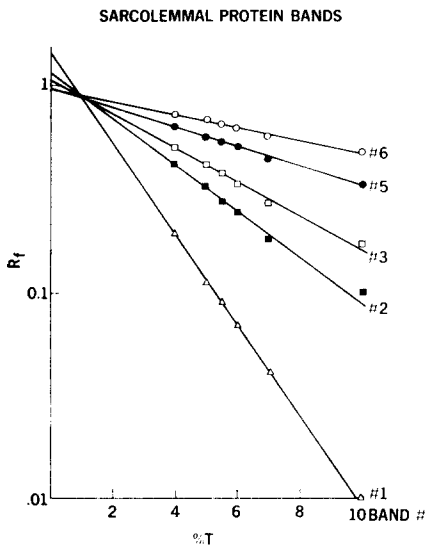

Fig. 11. Ferguson plots showing the relative mobility (R_f) versus the percent total gel concentration (% T) for major sarcolemmal membrane proteins shown in Fig. 10. Note the convergense of all the lines indicating similar mobility at "zero" gel concentrations. The numbers on the lines refer to the protein components shown in Fig. 10.

by determining their mobility relative to the mobility of the dye marker after correction for gel length by the procedure described by Weber and Osborn [20]. The molecular weight determined in this manner ranged between 98 000 and 112 000, in different experiments on 5.6 % gels for the larger protein and 22 000–26 000 for the Ca^{2+}-stimulated smaller component. It was pointed out [21, 22] that the use of relative mobility in sodium dodecyl sulfate gel electrophoresis to estimate molecular weight of a protein is valid only if its mobility and that of the reference proteins are the same at "zero" gel concentration. Therefore, we examined the consistency of the migratory pattern of phosphoproteins at different gel concentrations. The Ferguson plot [23] (Fig. 11) of relative mobility (R_f) versus percent total gel concentration (% T) indicates that different membrane proteins, including the major phosphoprotein (band 2) have nearly identical mobility at "zero" gel concentration. Similar Ferguson plots were also obtained (not shown) for reference proteins. These experiments suggest the validity of molecular weight estimation in our electrophoresis conditions. In subsequent experiments using the computer program developed by Rodbard and Chrambach [22] we obtained molecular weights of $108\,000 \pm 8\,000$ for the larger component and $25\,000 \pm 2000$ for the smaller component. Similar molecular weights were obtained using 3 types of calibration curves, R_F vs. log M_r, M_r vs. log R_F or K_R (retardation coefficient) vs. M_r.

Chemical characterization of Na^+-stimulated ^{32}P intermediate

The phosphorylated membrane complex was found to be highly sensitive to alkaline pH and acidified hydroxylamine. The effect of pH upon the liberation of ^{32}P from trichloroacetic acid-treated, ^{32}P-labeled membranes is shown in Fig. 12a. The hydrolysis of the Na^+-stimulated phosphoprotein becomes pronounced as the pH was

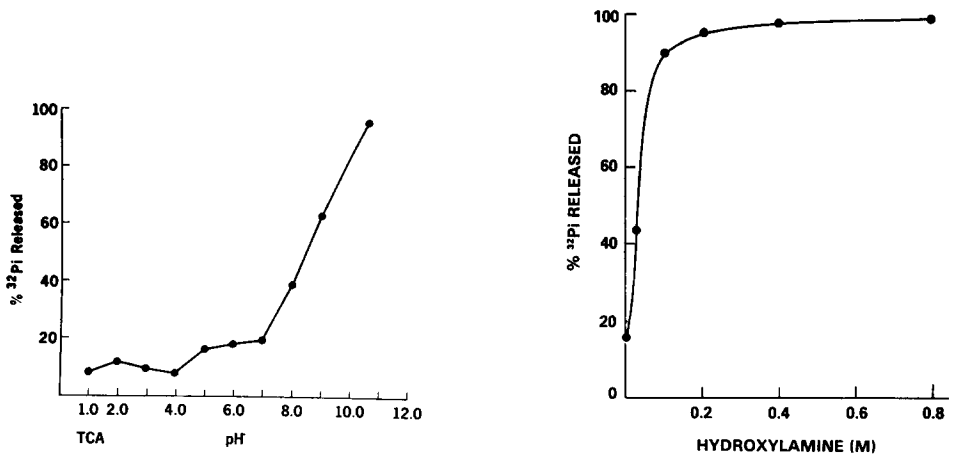

Fig. 12. pH stability (A) and hydroxylamine sensitivity (B) of phosphoprotein. Sarcolemmal membranes (4–6 mg) were incubated in a medium containing 25 mM Tris/HCl pH 7.5, 0.1 mM [γ-^{32}P] ATP, 1 mM Mg^{2+} and 100 mM Na^+ in a total volume of 2 ml at 0° for 10 s. The reaction, conducted in 30 ml polycarbonate ultracentrifuge tubes, was stopped by adding 20 ml of an ice-cold stopping solution. The tubes were centrifuged at 25 000 × *g* for 30 min at 4 °C. The pellets were resuspended and washed twice with 25 ml of stopping solution and once with ice-cold distilled water. The washed pellets were resuspended by homogenization in 4 ml of distilled water. Aliquots (0.2 ml) were incubated either with different buffer solutions: 0.1 M KCl/HCl (pH 2.0), 0.1 M citrate (pH 3 and 4), 0.1 M succinate (pH 5 and 6) and 0.1 M Tris/HCl (pH 7–10.5) or with 0.1 M sodium acetate containing different concentrations of hydroxlamine (pH 5.4). The samples (final volume 1 ml) were incubated for 30 min at room temperature. The reaction was stopped by the addition of 2 ml of ice cold 20 % trichloroacetic acid. After centrifugation the radioactivity in both the supernatant and pellets was determined as described in Methods.

raised above 7.0 while relatively little decomposition occurred between pH 2.0 and 6.5. Similar results (not shown) were also observed for the membranes phosphorylated in the presence of Mg^{2+} alone. The pH lability curve is consistent with the possibility that an acylphosphate bond may be involved as has been suggested for the phosphoprotein intermediate of (Na^++K^+)ATPase [9]. We further tested the stability of phosphoprotein to hydroxylamine treatment, a procedure known to hydrolyze the acyl phosphate bond [24]. When the phosphorylated sarcolemmal membranes were exposed to acidified hydroxylamine as much as 94 % of the incorporated phosphate was released (Fig. 12b). Similar exposure to pH buffer control and KCl ionic strength control revealed negligible phosphate release. Mg^{2+}-stimulated ^{32}P incorporation was equally sensitive to hydroxylamine treatment (data not shown).

DISCUSSION

The results of the present study provide evidence that the major phosphorylation activity of sarcolemmal membranes has many features in common with the phosphorylated intermediate of (Na^++K^+)ATPase reported extensively for other tissues [9, 10]. Thus a principal finding in this study is that a phosphorylated protein intermediate of sarcolemmal (Na^++K^+)ATPase can be identified and purified by sodium dodecyl sulfate gel electrophoresis. The molecular weight of the phosphoprotein is

found to be about 108 000, which is similar to that reported in the literature for the $(Na^+ + K^+)$ATPase phosphoprotein from other sources [9, 10]. The phosphoryl acceptor protein constitutes a dominant protein band of sarcolemmal on sodium dodecyl sulfate gels. Though not calculated, by inspection this protein band represents a considerable percentage of the total sarcolemmal protein. These findings might be expected of such a molecule underlying the unique and important function of cation transport across the muscle fiber membrane. Since we observed phosphorylation of a similar component under varied ionic conditions (Mg^{2+}, $Mg^{2+} + Na^+$) it may indicate that the same protein moiety serves as an acceptor for phosphate binding. Whether this protein is a single homogenous component or an aggregate of several components having similar electrophoretic mobility is not known.

The remarkable similarities between the characteristics of membrane bound $(Na^+ + K^+)$ATPase and the ATP-dependent sodium pump in intact cells lead to the belief that this enzyme system is identical with or closely related to the active Na^+ and K^+ transport across cell membranes [9, 10]. Currently available evidence suggests that a Na^+-specific phosphorylation of a membrane component by ATP initiates a reaction sequence that directly or indirectly participates in ion translocation, perhaps through conformational and/or other transformations. A K^+-stimulated dephosphorylation of the membrane system is thought to complete the cycle allowing its return to the lower energy state ready for the next cycle.

Available information from extensive isotopic flux studies suggests that sodium efflux in skeletal muscle is realized by three different mechanisms; exchange diffusion, active transport and passive leak [5]. The molecular entities of skeletal muscle membranes associated with these processes remain to be elucidated. In this respect the $(Na^+ + K^+)$ATPase, a surface membrane component, is of special importance in skeletal muscle because of its possible role in maintaining the transmembrane Na^+ and K^+ gradients that underlie the excitability and subsequent contraction. However, the properties of skeletal muscle $(Na^+ + K^+)$ATPase are relatively less understood and much less so in altered physiological and pathological conditions.

Several reports [11, 12, 25–29] in the recent past, utilizing different isolation procedures, suggested that the $(Na^+ + K^+)$ATPase of skeletal muscle is predominately recovered in a sarcolemmal membrane fraction. The results presented in this paper further indicate that a phosphorylated intermediate is associated with $(Na^+ + K^+)$-ATPase system of sarcolemmal membranes. The specific Na^+ requirement for its formation (Fig. 2) and K^+ for decomposition (Fig. 8) is highly suggestive of such function. Its requirement for Na^+ showed saturation kinetics (Fig. 2). The phosphorylation reaction was quite rapid (Fig. 1) and initial velocities could hardly be detected. A recent study utilizing a rapid mixing device revealed that the steady state level of phosphorylation of brain $(Na^+ + K^+)$ATPase preparation is achieved within a few milliseconds [25]. The amount of ^{32}P intermediate corresponds to the $(Na^+ + K^+)$-ATPase activity (Figs 6. 7) and the intermediate showed a high rate of turnover. The turnover number of about 12 000 obtained in the present study is of the order of magnitude reported for other particulate $(Na^+ + K^+)$ATPase preparations [9, 10] and further suggests its functional importance in the overall reaction sequence. The particulate subfraction obtained by sucrose density separation (SLF_1) appears to represent a relatively purified membrane preparation which possessed higher levels of $(Na^+ + K^+)$ATPase and its ^{32}P intermediate compared to the crude sarcolemmal

fraction. Nevertheless, phosphorylation of this fraction (SLF_1) was not influenced either by cyclic AMP or by cyclic GMP suggesting that the isolated sarcolemma contain no significant level of protein kinase activity which was cyclic nucleotide dependent. Furthermore, basal Mg^{2+}-stimulated phosphorylation was sensitive to hydroxylamine suggesting that cyclic nucleotide independent activity is also absent from these sarcolemmal membranes.

In summary, we have shown that sarcolemmal membranes are phosphorylated on brief incubation with ATP. The phosphorylation is primarily influenced by cations and the phosphoprotein formed bears the characteristics of an acylphosphate. The size of the phosphoprotein is approx. 108 000 daltons. These observations constitute the demonstration of a phosphorylated intermediate of sarcolemmal $(Na^+ + K^+)$-ATPase which has not been previously reported. The characteristics of the sarcolemmal ^{32}P intermediate suggest that the mechanism(s) of active cation transport by $(Na^+ + K^+)$ATPase system in skeletal muscle may be similar to that suggested for other tissues and erythrocytes [9, 10]. Such an idea is further supported by a recent study [31] in which it was shown that the binding of ouabain, a potent blocker of Na^+-pump activity, to intact muscle cells was qualitatively closely similar to that described for microsomal $(Na^+ + K^+)$ATPase preparations. We are currently investigating the [^{3}H]ouabain-binding properties of the isolated sarcolemma in order to elucidate further the characteristics of skeletal muscle $(Na^+ + K^+)$ATPase in normal and pathological tissues. Of considerable interest is our observation that [^{3}H]ouabain binding, $(Na^+ + K^+)$ATPase and (Mg^{2+})ATPase all increase in sarcolemmal membranes after denervation of both fast and slow muscles (Festoff, B. W, Oliver, K., and Reddy, N. B., submitted). The Na^+ stimulation of ^{32}P incorporation, however, does not change, suggesting that the turnover number increases considerably after depriving the muscle of its neural influence. It is possible that the known alterations in muscle membrane physiology in certain experimental as well as human pathological conditions may involve this protein intermediate at the molecular level.

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