Polypeptide Components of an Excitable Plasma Membrane*

STUART P. GREFRATH AND JACQUELINE A. REYNOLDS

From the Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710

SUMMARY

The excitable axonal membrane from garfish olfactory nerve has been purified by density gradient centrifugation using a modification of the Ficoll-sucrose method of Day et al. (Day, E. D., McMillan, P. N., Mickey, D. D., and Appel, S. H. (1971) Anal. Biochem. 39, 29). Three fractions are obtained with densities ranging from 1.051 to 1.064 g per ml. The membrane contains approximately 28% protein and the polypeptide chain composition has been analyzed on polyacrylamide gels in the presence of sodium dodecyl sulfate. The phosphorylated intermediate of the (Na⁺-K⁺)-ATPase has a molecular weight of 100,000 and the (Na⁺-K⁺)-ATPase activity is more than 90% of the total ATPase activity in the membrane.

We report here the purification of the axonal membrane from garfish olfactory nerve by density gradient centrifugation and the analysis of its polypeptide chain composition. In effect, this represents the first such analysis for a purified excitable membrane, and it is reasonable to expect that all of the major polypeptide chains observed are in some way related to neuronal function. We have identified one of the chains as a component of the (Na⁺-K⁺)-ATPase of the membrane and have noted that this enzyme constitutes more than 90% of the total ATPase activity associated with this system, a much larger fraction than previously reported for unpurified axonal membranes.

EXPERIMENTAL PROCEDURE

Longnose gar (Lepisosteus osseus) were purchased frozen from Gulf Specimen Co., Panacea, Fla. The olfactory nerve was removed according to the procedure of Easton (4) with the exception that the nose was cut approximately 1 cm posterior to the olfactory pits and the pit region was discarded so that the neuronal cell bodies were no longer present. The nerves were washed once in 10.5% sucrose-0.1 M NaHCO₃, pH 7.95, and then teased and washed twice in the same solution. The nerve was blotted, weighed, and homogenized gently (5 to 10 strokes by hand) in 10 volumes (v/g) of Ficoll-sucrose (density = 1.04396 g per ml)-0.1 M NaHCO₃ in a Thompson glass-Teflon homogenizer. The homogenate was layered on a Ficoll-sucrose step gradient prepared according to Day et al. (5) and centrifuged at 20,000 rpm in a Beckman SW 41 rotor at 5°C. The density steps were 2 ml each, corresponding to 1.04396, 1.05550, 1.05942, 1.06376, 1.12276, and 1.17272 g per ml. The resulting membrane fractions were washed four to five times with 10.5% sucrose-0.1 M NaHCO₃ and the pellets were collected at 48,000 × g in a Sorvall SS 34 rotor, 30 min, 5°C.

Protein was determined by the method of Lowry et al. (6) and phosphate was measured according to Bartlett (7). ATPase activity was measured in a 0.5-ml assay solution containing 10 mM imidazole-HCl (pH 7.4), 0.4 mM EDTA, 2 mM MgCl₂, 40 mM NaCl, 20 mM KCl, 2 mM ATP, and 5 to 15 μg of membrane protein. The amount of inorganic phosphate produced in 30 min at 37°C was determined by the method of Taussky and Schorr (8). (Na⁺-K⁺)-ATPase activity was taken as the difference in activity in the presence and absence of 0.5 mM strophanthidin (International Chemical and Nuclear Corp., Irvine, Calif.). SDS-polyacrylamide gel electrophoresis was carried out according to the procedure of Weber and Osborn (9).

[γ-³²P]ATP (New England Nuclear Corp., Boston, Mass.) was used to label the phosphorylated intermediate of the (Na⁺-K⁺)-ATPase, and the isolated membrane fractions were subjected to SDSD-Polyacrylamide gel electrophoresis to determine the polypeptide chain composition of the membrane. The membranes were isolated from the accompanying blood vessels, connective tissue, and myelinated trigeminal nerve. The synaptic connections occur only at the ends of the 15- to 20-cm-long axons and can be easily removed. It is thus possible to obtain an uncontaminated preparation of neurons for analytical and biochemical studies.

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† The abbreviation used is: SDS, sodium dodecyl sulfate.
K⁺-ATPase. Membrane vesicles containing 50 to 100 μg of protein were incubated at 25°C in a solution containing 16.2 mM Tris-HCl, 8 mM HCO₃⁻, pH 7.6, 3.2 mM MgCl₂, 100 to 120 mM NaCl, 4 to 6 μM [γ-³²P]ATP, and 0 or 40 mM KC1 in a total volume of 104 μl. The reaction was stopped after 10 s by the addition of 10 μl of 10% SDS, β-Mercaptoethanol (145 mM), 5 μl, was added to the final solution and the entire sample was subjected to SDS polyacrylamide gel electrophoresis. Gels were sliced in 2-mm sections and each sample was counted in a Packard Tri-Carb liquid scintillation counter using toluene-based scintillation fluid.

Lactate dehydrogenase activity was measured by the method of Kornberg (10).

RESULTS

Preparation of Axonal Membrane—Table I presents representative analytical data for the five fractions collected from density gradient centrifugation of the homogenized, dissected nerve tissue. Eight different preparations showed no significant deviation from this pattern. The supernatant corresponding to a solution density of 1.04396 g/ml contained soluble protein and the majority of the lactate dehydrogenase activity. This soluble cytoplasmic enzyme was investigated in an attempt to ascertain the possible level of contamination by water-soluble proteins in the various membrane fractions which arise through entrapment during homogenization and washing. Published data on the specific activities of a variety of lactate dehydrogenases from dogfish, tuna, and halibut (11) were used to estimate the number of milligrams of lactate dehydrogenase present in the membrane fractions. It can be seen from Table I that this is a very small percentage of the total protein in each fraction.

The three membrane fractions at densities of 1.05104, 1.05942, and 1.06376 g/ml were identified as axonal plasma membrane. They represent 64% of the total membrane weight as shown in Table I. (Approximately 75% of the total membrane in garfish olfactory nerves has been estimated as axolemma (3), the remainder being mitochondria and Schwann cell.) Furthermore, as is discussed in the following sections, these fractions contain 63% of the total Na⁺K⁺-stimulated ATPase activity and this activity is ≥90% of the (Mg⁺⁺-Na⁺K⁺)-ATPase activity in these purified membranes as would be expected for excitable membrane. The polypeptide chain compositions of all three fractions are closely similar. Electron microscopic examination of these membrane fractions showed closed vesicles and no observable mitochondria. Refloation of each of the axonal membrane fractions resulted in a single band at the original density. The heterogeneity in density observed for the axolemma probably arises from a heterogeneity in protein and lipid distribution along the 15- to 20-cm length of the membrane of the single nerve cell. Homogenization leads to rupture of the membrane and the formation of small vesicles which are formed from the membrane fragments. This heterogeneity has also been observed in myelin (5) and in squid retinal membrane (12).

The heaviest bands occurring at 1.12372 and 1.17272 g/ml were extremely diffuse, loosely packed, and incompletely separated. They correspond in density to inner, outer, and intact mitochondria (1.21, 1.124, and 1.173 g/ml, respectively) and have a phospholipid to protein ratio of 0.68, close to that found in mitochondria in other systems. Since the garfish had been frozen and thawed prior to dissection, there is a possibility of osmotic rupture of the mitochondrial components which could account for the loose packing in this density region. Small amounts of connective tissue and other cellular debris also appear in the highest density portion of this fraction at the bottom of the gradient.

Membrane Composition—An examination of Table I reveals that 63% of the total (Na⁺K⁺)-ATPase activity is associated with the three plasma membrane fractions. The specific activity of this enzyme, calculated as micromoles of inorganic phosphate released per mg of membrane protein per hour, is nearly constant in the two lighter density fractions at 28.8 and 25.8, respectively. Particularly noteworthy is the high percentage of total ATPase activity which is Na⁺K⁺-stimulated. Unpurified squid retinal axons, for example, show only 40% of the total ATPase activity as Na⁺K⁺-stimulated (12).

Approximately 27% of the (Na⁺K⁺)-ATPase activity is found in the heaviest density fraction which contains mitochondria and small amounts of connective tissue and other cellular debris. Some of the plasma membrane may also be coedinating in this region as the result of either entrapment or density heterogeneity. Since the axolemma is estimated as 75% of the total nerve membrane (3) and 64% is found in the lighter density fractions, less than 15% of the total plasma membrane could be present in the density region between 1.12372 and 1.17272 g/ml.

The lipids from fractions at 1.05104 and 1.05942 g/ml were extracted into chloroform-methanol (2:1) solution and examined on thin layer chromatography using two-dimensional elution. Chloroform-methanol-28% NH₄OH in the ratio 65:25:5 was used in the first direction, and chloroform-acetone-methanol-acetic acid-water in the ratio 3:4:1:0.5 was used in the second dimension. The lipid pattern was nearly identical with that observed by Chacko et al. (13) for whole garfish nerve with phosphatidylethanolamine predominating. Sphingomyelin, phosphatidylserine, and phosphatidylinositol were present in lesser amounts. The presence of cholesterol was noted at the solvent front, and three glycolipids were observed near the origin corresponding to those reported by Holton and Easton (14) in whole nerve.

### Table I

| Density (g/ml) | Weight per cent of total nerve membrane | Protein (mg) | Ratio of phospholipid to protein | Total ATPase (μmol/min/mg protein) | (Na⁺K⁺)-ATPase (μmol/min/mg protein) | Lactate dehydrogenase (μmol/min/mg protein) |
|---------------|----------------------------------------|-------------|---------------------------------|-----------------------------------|--------------------------------------|---------------------------------------------|
| 1.04396       | 10.55                                  | 0.325       | 4.64                            | 4.64                              | 1.00                                 | 0.010                                       |
| 1.05104       | 12.2                                   | 2.33        | 42.4                            | 38.9                              | 0.92                                 | 0.0002                                      |
| 1.05942       | 50.5                                   | 0.46        | 187.0                           | 103.1                             | 0.91                                 | 0.0009                                      |
| 1.06376       | 1.5                                    | 0.32        | 7.8                             | 4.13                              | 0.89                                 | 0.0001                                      |
| 1.12372       | 35.8                                   | 0.68        | 125.5                           | 116.9                             | 0.77                                 | 0.0003                                      |
| 1.17272       | 63.8                                   | 0.68        | 125.5                           | 116.9                             | 0.77                                 | 0.0003                                      |

* Calculated using a phospholipid to protein ratio of 25.

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* Microsomes of P₁ per mg of protein per hour times milligrams of total protein.

* Strophanthidin-sensitive ATPase: microsomes of P₁ per mg of protein per hour times milligrams of total protein.

* Calculated assuming a specific activity of 20,620 μmoles per mg per hour (see text).
Assuming that this is a reasonable figure for the purified membrane, which is 75% of the total weight in the whole nerve, one obtains a total lipid weight of 20.6 mg. Thus, the composition of the purified membrane can be estimated at 28% protein, 72% lipid. In a partially purified preparation of retinal axonal membrane from the squid, Fischer et al. (12) found 37% protein by weight. It would appear that highly purified excitable axonal membranes contain less protein than a common plasma membrane such as that from the erythrocyte (50% protein) and are closer to myelin in composition (20% protein).

**Polypeptide Composition**—The polypeptide chain composition for the three axonal membrane fractions using 7.5% polyacrylamide gels in sodium dodecyl sulfate is shown in Figs. 1 to 3. Approximately equal amounts of protein were applied to each gel. The principal polypeptide components are present in all three fractions but in slightly different ratios. Table II presents the molecular weights of the major components determined with three different concentrations of acrylamide in SDS polyacrylamide gel electrophoresis. There is no significant difference in molecular weights as observed in the three different systems. This correspondence gives confidence to the correct assignment of molecular weights since incorrect hydrodynamic size or incorrect SDS binding will be reflected as a variation in apparent molecular weight as a function of acrylamide concentration (15). In addition, a small amount of the axonal membrane was dissolved in 6 M guanidine hydrochloride, reduced and alkylated, and passed down a 6% agarose column as described previously for myelin and erythrocyte ghosts (16, 17). The resolution is extremely poor in this system. However, a major broad and asymmetric peak was observed at 45,000 to 50,000 daltons and a smaller peak at 35,000 daltons. The 100,000-molecular weight polypeptide appeared as a shoulder on the void volume peak which contained lipid vesicles. Within experimental error the molecular weight classes deduced from examination of polypeptide chains in the random coil form generated by the use of 6 M guanidine hydrochloride are the same as those deduced on the basis of electrophoresis of SDS-protein complexes.

In SDS-7.5% polyacrylamide gels which were electrophoresed for a shorter period of time than those shown in Figs. 1 to 3, a white, fluorescent lipid-SDS mixed micelle was observed running ahead of the tracking dye which stained heavily for carbohydrate and demonstrates again the existence in this preparation of the glycolipid reported by Chacko et al. (13) on whole garfish nerve tissue.

**Phosphorylated Intermediate of the (Na+K+)-ATPase**—Suspensions of the plasma membrane fractions were incubated in the presence of 100 mM Na+, 3.2 mM Mg2+, and 4 μM [γ-32P]-ATP. Since K+ increases the dephosphorylation rate of the phosphoenzyme intermediate of this ATPase (18), a control was run by including 40 mM K+ in the medium. After 10 s the reaction was stopped by addition of SDS as described under “Experimental Procedure,” and the entire solution was electrophoresed on a 5% polyacrylamide gel. The gels were stained with Coomassie blue and scanned before slicing into 2-mm sections. This permitted an accurate determination of molecular weight of the radioactively labeled polypeptide. Destaining gels prior to counting also served to remove the unreacted [γ-32P]ATP. The results for a typical labeling experiment are shown in Fig. 4.
brane contains approximately 28% protein by weight, which makes this a relatively lipid-rich membrane. The gel pattern of polypeptide chains is relatively simple and contains polypeptide chains ranging from 32,000 to 150,000 daltons. One of the chains of molecular weight about 100,000 has been shown to be the phosphorylated intermediate of the (Na\textsuperscript{+},K\textsuperscript{+})-ATPase in agreement with other data on this enzyme in different systems (see, for example, Refs. 23 and 24).

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FIG. 4. Electrophoresis on 5% polyacrylamide gel (0.1% SDS, pH 7.2 phosphate buffer) of phosphorylated polypeptide chain of the (Na\textsuperscript{+},K\textsuperscript{+})-ATPase. ——, 0 K\textsuperscript{+} added; ——, 40 m\textsuperscript{m} K\textsuperscript{+} added.

The single labeled polypeptide chain corresponds to 100,000 daltons and, as would be predicted, no label was seen in the control containing 40 m\textsuperscript{m} K\textsuperscript{+}.

DISCUSSION
The present work illustrates dramatically the need to isolate excitable membranes from systems that are nonmyelinated. The garfish olfactory nerve membrane has a density ranging from 1.051 to 1.064 g per ml. The myelin from the trigeminal nerve has been shown in this laboratory to have a density of 1.051 g per ml and Day et al. (5) have obtained three myelin fractions from rat brain with densities of 1.054, 1.060, and 1.066 g per ml. The severe overlap in density gradient centrifugation of the excitable membrane with myelin demonstrates the great difficulty in obtaining pure fractions from the vast majority of nervous tissue.

Material that is purified as we have described should give experimental results more nearly representative of excitable membranes per se than previously published work. This is illustrated by our finding that 90% of the ATPase activity in our preparations can be inhibited by strophanthidin, whereas only 40% inhibition has been reported for less pure preparations (12). The results suggest that recent binding studies of specific neurotoxins to garfish olfactory nerve (19-21) and other excitable systems (22) may be misleading since whole nerves or homogenates were used.

We have found that the garfish olfactory nerve axonal membrane contains approximately 28% protein by weight, which
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