Proc. Nat. Acad. Sci. USA
Vol. 70, No. 4, pp. 1191–1194, April 1973

Na^+-Selective Ionophoric Material Derived from Electric Organ and Kidney Membranes

(Electrophorus/black lipid membranes/peptides)

ADIL E. SHAMOO AND R. WAYNE ALBERS

Department of Physiology and Biophysics, Mt. Sinai School of Medicine of the City University of New York, New York N.Y. 10029; and Laboratories of Biophysics and Neurochemistry, National Institute of Neurological Diseases and Stroke, National Institutes of Health, Bethesda, Maryland 20014

Communicated by Terrell L. Hill, February 5, 1973

ABSTRACT **Material that increases black lipid-membrane (oxidized cholesterol) conductance has been demonstrated in the acid-soluble fraction of tryptic digests of membrane fractions from Electrophorus electric organ and beef kidney. The conductance change elicited by this material is highly selective for Na^+. The activity of the material was greatly enhanced by passage through DEAE-cellulose. Activity could be destroyed by further incubation with Pronase. Since conductivity increases exponentially with dose of ionophore, the conductive unit may be an oligomer.**

Coupling of ion transport to metabolic energy sources evidently requires that the internal energy of the source (e.g., ATP) be transferred to a macromolecular system that also interacts with the ion to produce the active transport of the ion. The argument in support of a stoichiometric coupling of the energy and carrier functions at the molecular level and for the cyclic nature of these molecular "machines" has been discussed (1, 2).

Considerable experimental evidence exists for the transfer of energy from a high-energy phosphate bond of ATP to an acceptor protein in the form of a phosphorylated intermediate, which is an integral component of plasma membranes (3, 4). Although the kinetics of Na^+ and K^+ activation of the ATPase reaction and of the active transport process have been described, little is known about the physical basis of the interaction of cations with the transport system.

The ($Na^+ + K^+$)-ATPase can be extracted from plasma membrane with the aid of certain detergents (5). It can be purified by extracting away other components of the membrane (6). It can be largely depleted of phospholipids (7). But in none of the above cases has the cationic requirement for ($Na^+ + K^+$)-ATPase activity been substantially altered. Accordingly, it is reasonable to suppose that the material that forms the sites of cation interaction may be an integral part of the macromolecular structure and quite possibly protein in nature. Although active sites of proteins are frequently the result of the tertiary protein structure, the existence of relatively small polypeptides with high affinities and specificities for alkali metal ions (8) is sufficient justification for a search for fragments of membrane-associated protein that may retain similar properties.

The black lipid membrane provides a convenient and sensitive assay system for ionophoric materials if they have some lipophilic character (9, 10).

Electrophorus electric organ constitutes perhaps the most active and readily prepared source of ($Na^+ + K^+$)-ATPase that is essentially free of other ATPases. Its properties have been extensively described (3).

We have recently reported the preliminary results of this work in two abstracts (11, 12). The work reported here demonstrates that acid-soluble material released by tryptic digestion of the Electrophorus ATPase preparation is capable of producing large conductance changes in black lipid membranes. These conductance changes are markedly specific in their requirement for Na^+. Comparable material has been obtained from bovine kidney, whereas peptides from bovine-serum albumin are essentially ineffective in this assay.

METHODS

Preparation and Tryptic Digestion of Membrane Fractions. The method for preparing eel electroplax membranes from Electrophorus has been described (13). The reaction mixture for tryptic digestion contained eel membranes equivalent to about 4 mg/ml of membrane protein and 10 μg/ml of trypsin in 40 mM Tris·HCl (pH 8) at room temperature (24°). The standard incubation time was 60 min*. The reaction was terminated by the addition of trichloroacetic acid to 5%. After centrifugation of the mixture for 45 min at 20,000 $\times$ g, the supernatant was removed and lyophilized. The dry material was dissolved in distilled water to 20% of the original volume. A portion of this material was diluted 5-fold and passed through a DEAE-cellulose column, which had been equilibrated with 0.2 M ammonium acetate at pH 5.1. The column volume was about 1 ml/ml of original reaction mixture. The column eluants were lyophilized and redissolved in a volume equivalent to 1 ml/30–40 mg of original membrane protein. Two types of control extracts were prepared. One consisted of the above reaction mixture prepared with trypsin inhibitor added before trypsin; the second control was a tryptic digest of an equivalent amount of bovine-serum albumin.

Beef-kidney membranes were prepared from a 1:2 homogenate of tissue in a solution consisting of 0.25 M sucrose, 1 mM ethylenediamine tetraacetic acid, and 0.1% deoxycholate (pH 7.6). After it was filtered through cheesecloth, the homogenate was centrifuged at 20,000 $\times$ g for 20 min. The supernatant was further centrifuged for 1 hr at 65,000 $\times$ g, the supernatant was discarded, and the final pellet was resuspended in 0.1 volume of deionized water. The tryptic digest

* It was found in more recent experiments that a 24-hr tryptic digestion increased the yield of the active material.

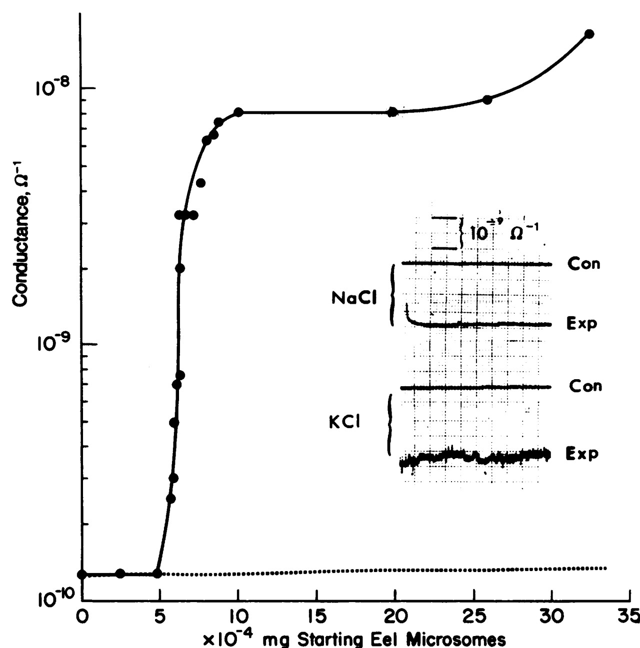

FIG. 1. Dose-response data for the addition of tryptic peptides from electric organ to the black lipid membrane assay. The tryptic digest had been "activated" by passage through DEAE-cellulose (see *text*). The *inset* illustrates the effects of high doses of tryptic peptides on the noise level of the membrane current measured at 50 mV. "Con" denotes current measured in the absence of peptides, "exp" denotes measurements taken 5 min after addition of peptides. "NaCl" denotes the conductance in tbe presence of Na^+ and the active material after a few minutes when the conductance had increased several-fold. "KCl" denotes the conductance in the presence of K^+ and the active material after 10 min. The *solid line* represents the conductance in the presence of Na^+ and the *broken line* represents the conductance in the presence of K^+.

procedure with this fraction was identical to that applied to the eel electric organ membranes. Pronase-digested control preparations were made by incubation of a portion of the DEAE-cellulose eluate equivalent to 10 mg of original membranes with 1 mg of Pronase at pH 8 for 18 hr at 25°. The reaction was terminated by boiling for 5 min and centrifuging 20 min at 20,000 × *g*. The lyophilized supernatants were made up in water as described above. Controls for Pronase were prepared similarly, except for the use of boiled Pronase.

Preparation of Black Lipid Membranes. Oxidized cholesterol was prepared by the method of Tien *et al.* (9). The membrane-forming solution consisted of 20 mg of oxidized cholesterol per ml of *n*-decane. The apparatus is essentially as described by Mueller *et al.* (10), as modified by Latorre *et al.* (14). The membrane diameter is 1 mm and the solution volume of the Teflon cup is 5 ml. By use of an electrolyte solution consisting of 0.1 M NaCl and 5 mM histidine (pH 7.0, and 19°), the black lipid membrane forms spontaneously within 1–3 min of application of the membrane-forming solution.

Conductance Measurements. The circuitry has been described (10, 14). The membrane was in series with a variable standard resistance (0.1–10 MΩ) and a voltage source; positive current and potential were defined to correspond to cation flow from the inner compartment. Material to be assayed was normally placed in the inner compartment. Additions were made either in less than 30-μl volumes or, if larger, with a compensatory addition of electrolyte in the outer compartment. Mixing in the inner compartment was done with a small, air-driven magnetic stirrer.

RESULTS

Black lipid membranes of oxidized cholesterol exhibit increased conductances upon the addition of tryptic peptides of electric organ membranes to the electrolyte solution, as indicated in Fig. 1. The activity of these preparations are expressed in terms of the protein content of the intact "microsome" fraction. Subsequent experiments show that about 15% of the protein is degraded to acid-soluble peptides in 60 min by our procedure. Thus, the major conductivity change is produced by less than 1 μg of material. The conductance of the membranes was initially about 10^{-8} Ω^{-1}/cm^2. Additions of active material to the inner compartment produced a rising conductance within 3 or 4 min that usually approximated a steady state within 5–10 min if the current flow was positive. If the current flow was negative from the start, there was no change in conductance, whereas if incorporation of the active material had been initiated by a positive current first then a negative current was applied later, the same steady state was attained after a much longer time interval. After a steady state was attained, variation of the voltage from 0 to 100 mV did not produce any abrupt conductivity changes as has been observed in the case of excitability inducing material, (EIM) (15).

The log–log plot of Fig. 1 indicates that the slope is 6–7. This may mean that 6–7 units of the active peptide are required to produce a conductive unit. This is similar to what is reported for alamethicin (16). The induced conductance change is selective for cations, as a Nernst potential of about 50 mV was observed for a 10-fold sodium salt gradient and this was positive on the low-salt side of the membrane. Fig. 1 also demonstrates the ineffectiveness of K^+ as an electrolyte in this system (*broken line*). Na^+ appears to be the only effective cation with respect to the increased steady-state conductivity induced by the electric-organ membrane peptides (Table 1). Some effects of the material could be seen with KCl as electrolyte, but these appeared mostly as an increased noise level (Fig. 1, *inset*). It should be made clear that the specific effect of Na^+ on the conductance pertains to in-

TABLE 1. *Cationic specificity*

Bathing solution (0.1 M) of*	Approximate fold increase in conductance of black lipid membrane
NaCl	300
KCl	1.0
LiCl	1.0
RbCl	1.0
CsCl	1.0
NH_4Cl	1.0

* In each solution there was also 5 mM histidine (pH 7.0). Original conductance = 10^{-10} Ω^{-1}. Dosage = 2 μg of starting eel microsomes. Number of experiments = 6. Fraction = Tryptic digest–Cl_3CCOOH–supernatant–DEAE-cellulose eluate (see *Methods*). Black lipid membrane = Oxidized cholesterol.

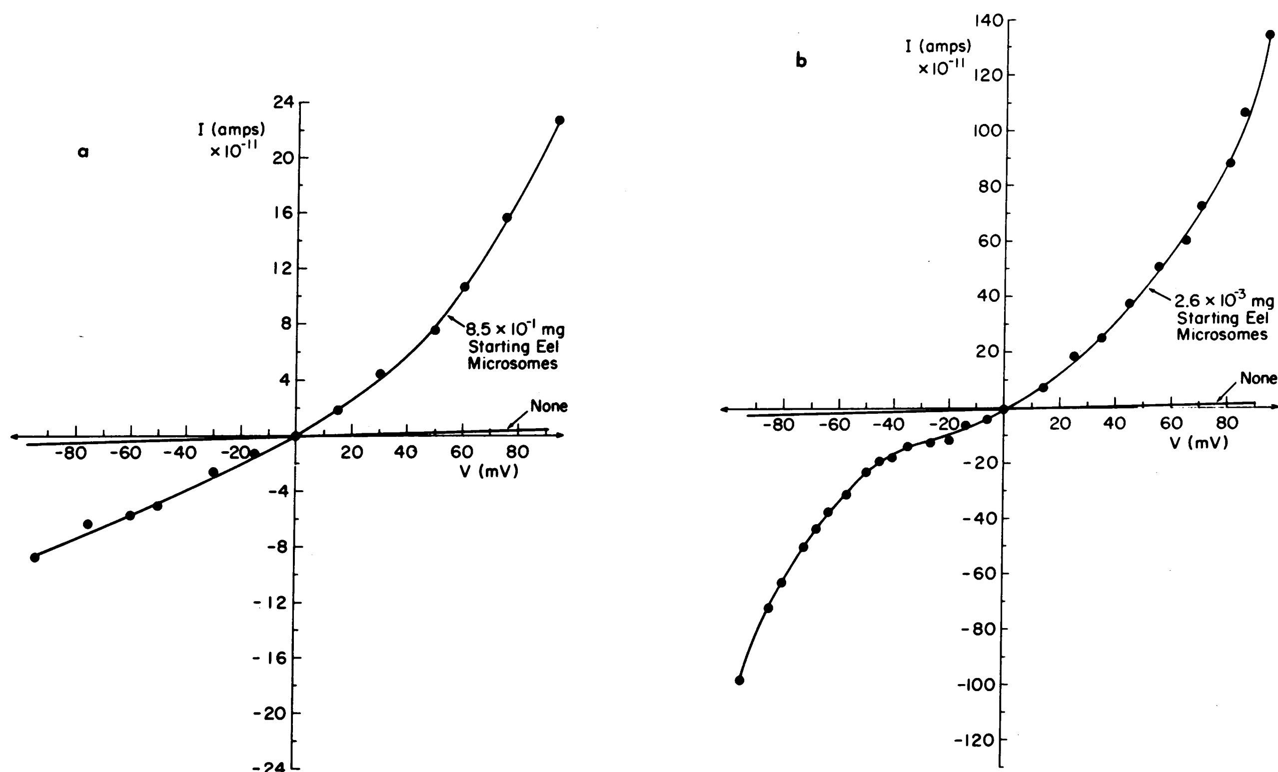

FIG. 2. Current-voltage relationship (*a*) in the presence of a fixed amount of tryptic digest and (*b*) in the presence of a fixed amount of tryptic digest activated by passage through DEAE-cellulose.

corporation of the active material into the bilayer. However, the specificity of the formed ionophores is not known and currently is under investigation. Competitive effects of K^+ seem to occur, however, as a suppression of the conductivity changes which can be induced by low concentrations of Na^+. Conductivity changes could be induced by addition of active material to 10 mM NaCl. No changes could be induced when the electrolyte was 10 mM NaCl + 100 mM KCl, but increasing NaCl to >100 mM permitted conductance changes to appear. At dosages of active material higher than 3.5 μg of starting eel microsomes, the conductance increases in the presence of 0.1 M KCl or 0.1 M $MgSO_4$. Thus, at high dosages of the material the membrane becomes leaky and nonspecific to the ions.

In experiments in which Na^+ and K^+ were present on opposite sides of the membrane, conductance changes were only observed when active material was added to the Na^+ side.

The "Na^+-conductance" property of the electric organ tryptic peptides was markedly enhanced by passage of the total acid-soluble fraction through a small DEAE-cellulose column (Fig. 2). Since trypsin preferentially hydrolyzes peptide bonds adjacent to arginine and lysine, it is not surprising that many of the acid-soluble fragments have basic properties such that they do not readily adsorb onto the column. As Table 2 indicates, similar preparations passed through cation-exchange columns were inactivated. Further evidence for the peptide nature of the active material comes from the observation that Pronase treatment inactivates the column-treated material but boiling does not. The beef-kidney preparation described above also produced increases in membrane conductance in this assay system. However, tryptic digests of bovine-serum albumin were ineffective except as they increased the noise level. Similar effects of these preparations could be obtained on black lipid membranes made from a solution of 7-dehydrocholesterol in decane or from a mixture of cholesterol and 7-dehydrocholesterol in decane. However, no such effects have been obtained with black lipid membranes prepared from total lipid extracts of electric organ with α-tocopherol or from lecithin.

DISCUSSION

Two types of conductivity changes have been reported, "carrier type" and "channel type," typified by valinomycin for "carrier type" and excitability-inducing material and

TABLE 2. *Effect of passage of the tryptic digest through different columns on the activity with respect to black lipid membrane conductance*

Eluate from column of:		Approximate fold increase in conductance of black lipid membrane
Anion-Exchangers	DEAE-cellulose	300
	Dowex 1-X2	205
	Ecteola-cellulose	125
Cation-Exchangers	Mannex-CM-cellulose	1.0
	Dowex 50-X8	1.0

Dosage = 0.3 μg starting eel microsomes. Bathing solution = 0.1 M NaCl + 5 mM histidine (pH 7.0). Number of experiments = 6. Fraction = Tryptic digest-Cl_3CCOOH-supernatant (see *Methods*).

alamethicin for "channel type." Direct observation of channels is possible at very low ionophore concentrations (14, 15). As the number of channels increases, the conductance characteristic becomes indistinguishable from the carrier type. Conductance jumps have been observed at low dosages of the whole tryptic digests and of the DEAE-cellulose eluate of electric organ. However, these jumps are not observed once the conductance increases beyond several-fold.

Once the conductance has increased beyond several-fold there is a dramatic increase in conductance when the current is positive. Thus, the positive voltage may be a requirement for whatever process is needed for the formation of the conductive unit. This process is similar to that reported for alamethicin (16). Conductance changes could be obtained in membranes containing cholesterol or dehydrocholesterol as the predominant constituents. Membranes prepared from total electric organ lipids or from soy-bean lecithin did not respond to these materials. The most obvious differences between cholesterol and phospholipid membranes are their structures at the aqueous interface. The ionic interface of phospholipid membranes may either prevent the entrance of charged polypeptides into the lipid phase or prevent their passage from one side to the other.

Although the tryptic digests that contain active material have so far only been prepared from $(Na^+ + K^+)$-ATPase preparations, this obviously does not demonstrate a necessary relationship. Several avenues are possible for further exploration of this point. Since the ATPase can be identified with two bands on sodium dodecyl sulfate–gel electrophoresis (5, 17), it will be of interest to prepare tryptic digests of these bands for use in the black lipid membrane assay. Most of the well-characterized ionophores either do not have a high Na^+/K^+ selectivity or are slective for K^+.

Recently, a polypeptide from the mushroom *Amanita* (antamanide) has been reported to have a high selectivity for Na^+ (18, 19). Its effects on conductivity of the black lipid membrane have not been reported. Goodall and Sachs (20) briefly reported that materials with both Na^+ and K^+ selectivity could be extracted directly from brain and electric organ. We have not found such activity in membrane fractions *per se*. However, these preparations have all been extensively washed to remove soluble material. Accordingly, it remains an interesting problem to investigate the possible relationship of the material that we have obtained with that obtainable by direct extraction of the whole tissue, as described by Goodall and Sachs. A soluble bacterial Mg^{++}-ATPase has been reported to increase conductance of the black lipid membrane (21). Jain *et al.* (22, 23) have reported that short-circuit current can be induced in the black lipid membrane in the presence of Mg^{++}, Na^+, K^+, ATP, and a brain synaptosome preparation. If this current is due to Na^+ or K^+, this could be taken as a reconstructed, active Na^+-transport system. Such a system still represents a high degree of biological organization. Elucidation of the molecular structure of the Na^+-carrier moiety of the system requires further dissection. The present investigation of tryptic digests of $(Na^+ + K^+)$-ATPase preparations represents an effort in this direction.

This study was supported in part by National Institutes of Health Training Grant AM0564202. We thank Dr. Harold Lecar for his valuable comments and discussion throughout the course of this work, and Michael Lincoln and Marjory Myers, Trainees at Mount Sinai School of Medicine of the City University of New York, for their valuable help in the biophysical and biochemical measurements.

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