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Na^+-Dependent Ionophore as Part of the Small Polypeptide of the $(Na^+ + K^+)$-ATPase from Eel Electroplax Membrane*

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Summary. The $(Na^+ + K^+)$-ATPase from eel electroplax membranes is resolved into two polypeptides by means of sodium dodecyl sulfate (SDS) preparative gel electrophoresis. From the literature, the larger polypeptide has been known to have a molecular weight in the range of 85,000 to 135,000, while the molecular weight of the smaller polypeptide is known to be between 40,000 and 60,000. When the two polypeptides are combined with a large/small molar ratio of 1:2, a very Na^+-dependent voltage-independent ionophoric activity is observed. The Na^+-specificity is manifested as a near absolute requirement for Na^+ in order for the two polypeptides to cause an increase in conductance. Further work with tryptic digests of the two polypeptides suggests that the Na^+-dependent ionophoric activity is associated with the smaller polypeptide of $(Na^+ + K^+)$-ATPase.

The role of the membrane-bound $(Na^+ + K^+)$-ATPase in active transport of Na^+ has been widely accepted (Siegel & Albers, 1970). A large volume of data suggests that this enzyme may mediate the transfer of energy from ATP-splitting to a "carrier-like" protein and to Na^+ ion. Thus, there may be a small peptide with high affinity and specificity for Na^+ that is an integral part of $(Na^+ + K^+)$-ATPase. The small peptide may be a small segment of a large polypeptide.

Several reports have recently indicated that proteins isolated from various tissue sources cause an increase in the conductance of a black lipid membrane. Substances with ionophoric activity have been derived from membranes of gastric epithelium (Sachs, Spenney, Saccomani & Goodall, 1974), cholinesterase (Jain, Mehl & Cordes, 1973), and eel electroplax $(Na^+ + K^+)$-ATPase preparation (Albers, Shamoo, Koval & Myers, 1973;

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Shamoo & Albers, 1973*a*, *b*; Shamoo, Myers & Albers, 1973; Blumenthal & Shamoo, 1974; Shamoo, Myers, Blumenthal & Albers, 1974).

We have recently published several reports (Shamoo & Albers, 1973*a*, *b*; Shamoo *et al.*, 1973; Blumenthal & Shamoo, 1974; Shamoo *et al.*, 1974) describing the isolation from an *Electrophorus* ATPase preparation of one or more ionophores requiring Na^+ for incorporation into a black lipid membrane preparation. This incorporation was demonstrated by measurement of an increase in bilayer conductance. The incorporation of the ionophore into the bilayer caused an increase in the conductance of the bilayer by a factor of 300. The ionophoric activity is defined in this report as an increase in bilayer conductance in the presence of material from the $(Na^+ + K^+)$-ATPase preparation.

In this report we show that a) an increase in conductance due to the presence of both polypeptides is faster in the presence of Na^+ as compared to the other monovalent cations, and b) in a weight ratio of 1:1 of small-to-large polypeptides of $(Na^+ + K^+)$-ATPase there is a voltage-independent increase in bilayer conductance. The increase in conductance is nearly absolute in its requirement for the presence of Na^+ as compared to the other monovalent cations.

Materials and Methods

Black Lipid Membranes and Ionophoric Preparations

The oxidized cholesterol membrane preparation and the electronic circuitry for conductance measurement of the black lipid membrane have been described previously (Shamoo & Albers, 1973*a*). The one-hour tryptic digestion and passage of the acid-soluble digest through a DEAE-column for each polypeptide was performed exactly in the same manner described previously for the microsomes (Shamoo & Albers, 1973*a*). The electrolyte solution on both sides of the membrane consists of 0.1 M salt (NaCl, KCl, etc.) and 5 mM histidine (pH 7.4, 19 °C).

SDS Preparative Gel Electrophoresis

The method for preparing eel electroplax membranes (rich in $(Na^+ + K^+)$-ATPase) from *Electrophorus* has been described (Albers *et al.*, 1963). The two polypeptides of $(Na^+ + K^+)$-ATPase were prepared in the laboratories of Dr. R. Wayne Albers (NIH) and provided to us for use as a lyophilized dry powder. The enzyme preparation was solubilized in 2% Lubrol, and a weight of asolectin (95% soya phosphatides, from Associated Concentrates, Long Island, N.Y.) equal to that of the weight of enzyme protein was added. The Lubrol-solubilized enzyme was then treated with 5% SDS (sodium dodecyl sulfate) at room temperature for 45 min. The SDS-solubilized enzyme (with no enzymatic activity) was run through 0.1% SDS preparative gel electrophoresis. The following SDS-preparative method (Jean & Albers, *J. Biol. Chem., in press*) of

gel electrophoresis was employed: The stacking gel consisted of 4% acrylamide 0.14% BIS (N,N′-methylene bisacrylamide), 0.24% TEMED (N,N,N′,N′-tetramethylethylene diamine), 0.1% SDS, 0.1% ammonium persulfate, 1 mg% riboflavin and 0.6 M sodium phosphate buffer, pH 7.1. The separating gel consisted of 5% acrylamide 0.175% BIS, 0.16% TEMED, 0.1% SDS, 0.2% ammonium persulfate, and 0.1 M sodium phosphate buffer, pH 7.1. The negative electrode upper chamber buffer consisted of 0.1 M sodium phosphate buffer, pH 7.1, and 0.1% SDS; the positive electrode lower chamber buffer consisted of 0.1 M sodium phosphate buffer, pH 7.1, and 0.1% SDS. The current was 50 mA with 35 V. The temperature was maintained at 16 °C. The surface area of the preparative gel was 8 cm^2. The composition of the SDS-analytical gel electrophoresis was the same as the preparative gel electrophoresis. The surface area was 0.196 cm^2; the current was 5 mA/tube, and the temperature was room temperature (21 °C). The protein fractions were dialyzed against distilled water for several days with anion exchange resin on the outside chamber. The samples were then lyophilized and stored in a desiccator at 0 °C as a dry powder. The protein concentration throughout this manuscript is expressed as a dry weight per ml of solution.

Results

Bilayer Conductance Changes Induced by the Polypeptides of $(Na^+ + K^+)$-ATPase

The $(Na^+ + K^+)$-ATPase preparation from the electric organ of *Electrophorus* consists of two polypeptides as resolved by SDS-gel electrophoresis (Collins & Albers, 1972; Jean & Albers, *J. Biol. Chem., in press*). The two polypeptides were similar in their migration pattern on analytical SDS-gel electrophoresis to those reported for canine renal medulla (Kyte, 1971*a*, 1972), shark rectal gland (Uesugi, Kahlenberg, Medzihradsky & Hokin, 1969; Hokin, Dahl, Deupree, Dixon & Perdue, 1973) and eel electroplax (Collins & Albers, 1972). For the various tissue sources (canine renal medulla, shark rectal gland and eel electroplax) the molecular weight determinations for the large polypeptide ranged between 85,000 to 135,000, and ranged between 40,000 to 60,000 for the small polypeptide. The identification of the two bands was made on analytical SDS-gel electrophoresis. In this report we will simply identify them by the letters *L* for the large polypeptide (85,200 daltons) and the letter *S* for the small polypeptide of $(Na^+ + K^+)$-ATPase (57,000 daltons) (Collins & Albers, 1972; Jean & Albers, *J. Biol. Chem., in press*). We would like to emphasize at this point that by no means are we implying that the two polypeptides are pure and contain no other bands. There may well be other small bands near them; therefore, further experimentation to assure the polypeptides' purity is needed.

Fig. 1 shows the protein patterns on disc gel SDS-polyacrylamide electrophoresis of the Lubrol-solubilized $(Na^+ + K^+)$-ATPase preparation

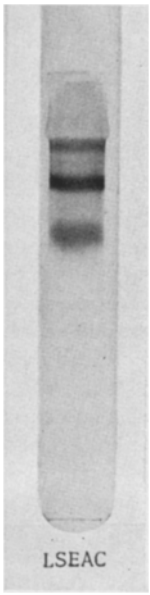

Fig. 1. Identification of the two polypeptides of the Lubrol-solubilized $(Na^+ + K^+)$-ATPase from eel electroplax on 5% acrylamide SDS-gel electrophoresis (*see* Materials and Methods for detail). The photograph was supplied by D. Jean and R. W. Albers

(*see* Materials and Methods). There are two major protein bands. The lower band is the small polypeptide of $(Na^+ + K^+)$-ATPase and the upper band is the large polypeptide of $(Na^+ + K^+)$-ATPase.[1]

Fig. 2 is a composite of several experiments. Each ionic condition represents an independent experiment on a bilayer. For each ionic condition, the data are representative of at least six different experiments. The variation from one experiment to another was in the magnitude of change (20 to 40%) in conductance in a given period of time.

As can be seen from Fig. 2 (*A*, *B* and *C*) the conductance was constant at $\sim 10^{-8}\ \Omega^{-1}/cm^2$ under all or any of the ionic conditions before the introduction of peptides into the bathing fluid of the bilayer. The conductance in the control period between 0 and 4 min was constant and displayed no rectification when voltage was changed from $+50$ mV to -50 mV. Rectification is defined as an asymmetric change in conductance due to

1 In more recent experiments for the isolation of the two polypeptides with analytical and preparative SDS-gel electrophoresis we employed the method reported by Laemmli (1970). Laemmli's method has been known to have a very high resolving power for protein separations. It was found that there were more than just two bands of the Lubrol-solubilized enzyme on the gel (8.75%), but rather several bands were close to each of the two polypeptides.

changes in the polarity of the applied voltage. The gaps in conductance measurement were a result of the time lost in the measurement of conductance due to the transient capacitative current.

The conductance data shown here, and in all of the Figures in this report, are average conductances. Noise in the conductance measurements amounted to a $\pm 3 \times 10^{-8}\ \Omega^{-1}/cm^2$ in the case of a $+50$ mV voltage clamp in the presence of various ions and any of the peptides or their tryptic digests. Noise level was very low ($< \pm 1 \times 10^{-8}\ \Omega^{-1}/cm^2$) in the case of a -50 mV voltage clamp.

Panel *A* represents the experiments in which at time four minutes we added about 5×10^{-4} mg/ml of either a) large polypeptide (*L*), b) small polypeptide (*S*), or c) tryptic digest of the large polypeptide. The polypeptides obtained after SDS-preparative gel electrophoresis and prolonged dialysis were lyophilized and stored in a desiccator at 0°C. The concentrations indicated are the weights of each material per ml of bathing solution inside the Teflon cup.

When the voltage was positive (*i.e.*, inside Teflon cup +), the current flowed from the inside of the Teflon cup toward the outside. Conductance then increased with any cation and any kind of peptide used (*see* Panels *A*, *B* and *C* between 4 and 6 min). When the voltage was negative (between 6 and 8 min) not only was no increase in conductance observed in the absence of Na^+, but also the negative potential either drove the ion-conducting material out of the membrane, or at least interrupted the conductive unit. When Na^+ was present with either the tryptic digest of *S* (Panel *B*) or the 1:1 weight ratio of *L* and *S* (Panel *C*), the conductance continued to increase slowly. When the voltage was returned to positive (between 8 and 10 min) there was again an increase in conductance under all of the ionic conditions and various peptide additions. However, when the voltage was negative, we observed that the conductance increased further only in the presence of Na^+ and the presence of either the tryptic digest of *S* (Panel *B*) or in the presence of both polypeptides together in a weight ratio of 1:1 (Panel *C*). In Panel *B* where the tryptic digest of *S* polypeptide was employed, conductance rectification was less pronounced in the presence of Cs^+, Rb^+ or NH_4^+ if the conductance was allowed to reach a steady state. Data not shown indicate that if the active ionophoric material is introduced during -50 mV voltage clamp, there will be a slow increase in conductance in the presence of Na^+ and no change in conductance in the presence of K^+, Cs^+, Rb^+, Li^+ or NH_4^+. Then when positive voltage is introduced a large increase in conductance will occur in the presence of any of the monovalent cations used, similar to the increase shown in Fig. 2. From

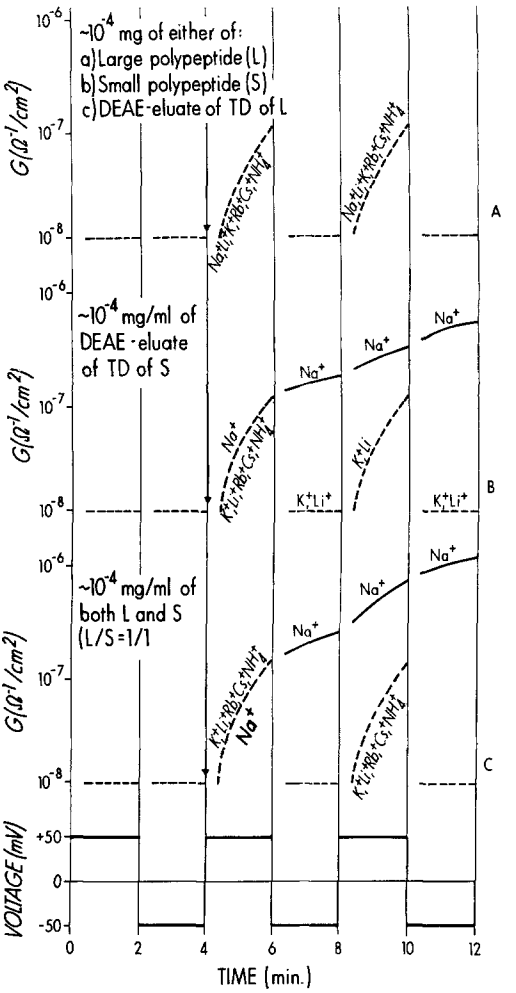

Fig. 2. Effect of polarity of voltage clamp on a black lipid membrane conductance in the presence of various polypeptides. The vertical axis represents conductance in Ω^{-1}/cm^2 in Panels *A*, *B* and *C* and voltage in mV in the lowest panel. The horizontal axis represents time in minutes for all the panels. At the period from 0 to 4 min for all three panels the bilayer bathing solution consisted of 0.1 M salt (either NaCl, KCl, LiCl, CsCl, RbCl or NH_4Cl) on both sides of the membrane and 5 mM histidine, pH 7.4, 19 °C. At $t=4$ min, we introduced into the bathing fluid inside the Teflon cup the following: For Panel *A*, $\sim 5\times 10^{-4}$ mg/ml of either a) large polypeptide (*L*) of $(Na^+ + K^+)$-ATPase, b) small polypeptide (*S*) of $(Na^+ + K^+)$-ATPase, or c) the one-hour tryptic digest of large polypeptide of $(Na^+ + K^+)$-ATPase; for Panel *B*, $\sim 5\times 10^{-4}$ mg/ml of the one-hour tryptic digest of small polypeptide (*S*) of $(Na^+ + K^+)$-ATPase; for Panel *C*, $\sim 5\times 10^{-4}$ mg/ml of both large and small polypeptide of $(Na^+ + K^+)$-ATPase together (i.e., weight ratio of 1:1). The voltage clamp for every 2 min was either +50 mV or −50 mV as indicated. The +50 mV indicates that the solution inside the Teflon cup is subjected to positive voltage with respect to the solution outside the Teflon cup. The polypeptides were at all times introduced into the inside of the Teflon cup. A positive potential produced a flow of a positive current from the inside bathing fluid, inside the

Fig. 2, one may conclude that when the voltage is clamped at -50 mV from $t = 0$, no increase in membrane conductance will be observed unless Na^+ is present with either *L* and *S* together or with the tryptic digest of *S*. The experiments did bear out such a conclusion. As a matter of fact, under no voltage at all (neutral) and with only small negative pulses every few minutes to measure the conductance, we observed results similar to those under negative voltage clamp conditions. The difference was that the time required to reach a maximum increase in conductances under zero voltage was approximately 20 min, whereas under negative voltage, the time was approximately 10 min. This may indicate that the negative voltage clamp speeds up either the formation or stabilization of the conductive unit within the bilayer.

The amount of SDS bound with either polypeptide was estimated to be 1 mole SDS per 1 mole of polypeptide (Kyte, 1972; Jean & Albers, *J. Biol. Chem., in press*). The addition of SDS alone at 10^{-4} mg/ml (maximum amount possible) had no effect on bilayer conductance under either positive, negative, or no voltage clamp. No measurements were made to estimate the amount of SDS bound to the tryptic digest fraction of either polypeptide, but it is safe to assume that it is no greater than that bound to each of the polypeptides.

Ionophoric Properties of the Tryptic Digest of the Small Polypeptide of $(Na^+ + K^+)$-ATPase

Fig. 3 is representative of six experiments made with each cation on the BLM conductance versus time in minutes. The conductance was continuously monitored under -50 mV voltage clamp. The $t = 0$ represents the electrolyte solution of 0.1 M salt (NaCl, KCl, etc.), 5 mM histidine, pH 7.4,

Teflon cup, to the chamber outside of the Teflon cup. The control period before the addition of any polypeptide was between 0 and 4 min. The broken lines in Panels *A*, *B* and *C* represent data where similar conductances were obtained in the presence of all monovalent cations employed. In Panel *B* the broken line after 6 min was shown only for K^+ and Li^+ because the inhibition of the increased conductance in the presence of these two ions was maximal as compared to the presence of Rb^+, Cs^+ or NH_4^+. In the presence of Rb^+, Cs^+ or NH_4^+ under the conditions of Panel *B*, the conductance would not be inhibited by the negative potential if the bilayer conductance reached a steady state. The solid lines in Panels *B* and *C* represent data where the conductances were unique in the presence of Na^+ only. The conductance measurements were made after the applied voltage had started and the transient capacitative current contribution was zero (0.2 min)

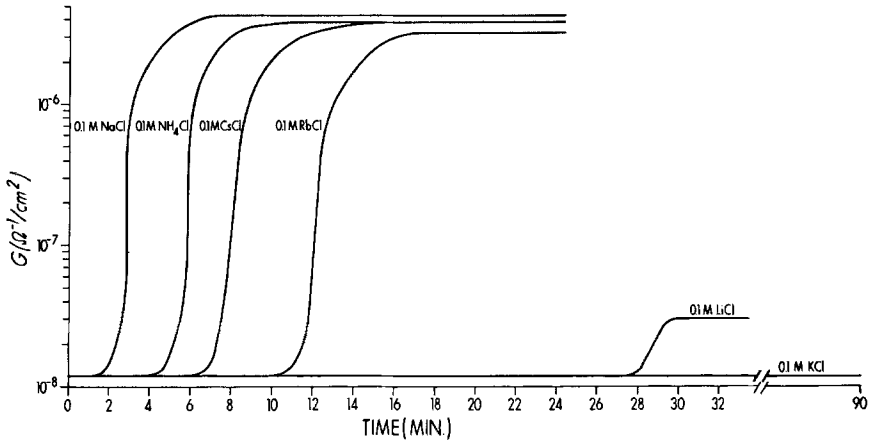

Fig. 3. The time response of a black lipid membrane conductance in the presence of various ions in the presence of $\sim 5 \times 10^{-4}$ mg/ml of the tryptic digest of the small polypeptide (*S*) of $(Na^+ + K^+)$-ATPase. The membrane was first formed, reached a steady state, then the tryptic digest was added at $t=0$ into the fluid inside the Teflon cup. The bilayer bathing fluid consisted of 0.1 M salt (either NaCl, CsCl, RbCl, LiCl or NH_4Cl as indicated), 5 mM histidine, pH 7.4, 19 °C. The conductance of the bilayer was continuously monitored under the condition of -50 mV voltage clamp. The fluid inside the Teflon cup which contained the ionophoric material was subjected to negative voltage with respect to the outside chamber

and in the presence of 5×10^{-4} mg/ml of the tryptic digest of the small polypeptide *S*. The bilayer was made a few minutes before the peptide was added.

The conductance of the black lipid membrane in the presence of Na^+ increased in a shorter time than in the presence of each of the other monovalent cations indicated (NH_4^+, Cs^+, Rb^+, Li^+ and K^+). From the data shown in this Figure and from the variability (20 to 40%) in the times required to reach maximum conductance, one could say only that the conductance increased in the presence of Na^+, NH_4^+, Cs^+ and Rb^+, and little or no increase in conductance was observed in the presence of Li^+ and K^+.

To demonstrate the greater effectiveness of Na^+ in increasing bilayer conductance, we performed the following experiment. First, we made the bilayer in the presence of 0.1 M KCl or LiCl, 5 mM histidine, pH 7.4. A few minutes later we added the tryptic digest of the small polypeptide *S*. The $t=0$ represents the time of addition of the small polypeptide. Fig. 4 shows the results of a representative experiment of such a design. The conductance was continuously monitored under -50 mV voltage clamp. No increase in bilayer conductance in the presence of K^+ and little increase

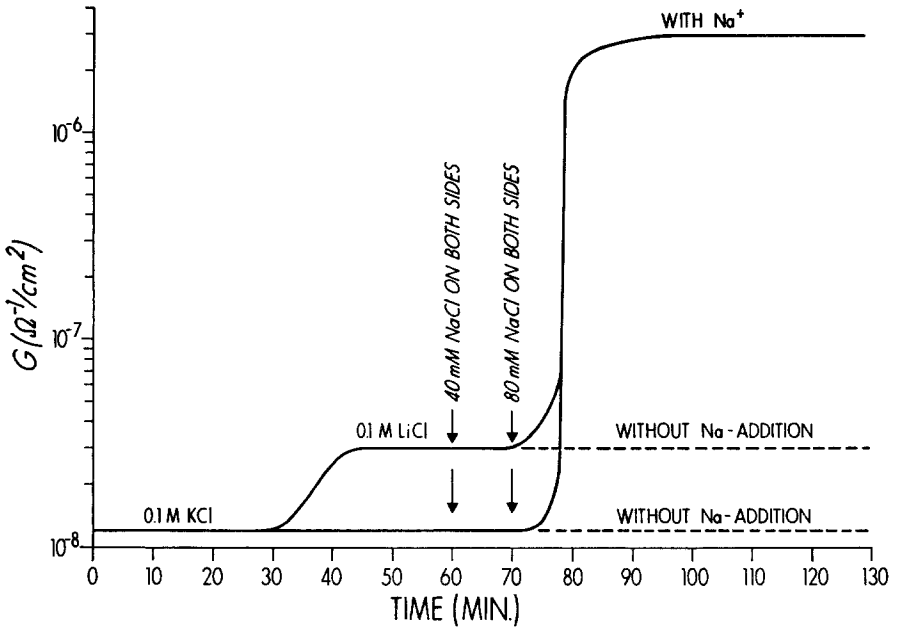

Fig. 4. The effect of Na^+ addition on time-response of conductance in the presence of $\sim 5 \times 10^{-4}$ mg/ml of the tryptic digest of the small polypeptide (*S*) of $(Na^+ + K^+)$-ATPase on a black lipid membrane conductance. The rest of the ionic and voltage conditions are the same as those described for Fig. 3, except the only two ions employed were K^+ and Li^+ (*see* text for details)

in conductance in the presence of Li^+ were observed. In both ionic conditions the addition of approximately 80 mM Na^+ on both sides of the membrane caused a dramatic increase in conductance equal to that observed in the presence of Na^+ alone. Neither the addition of Na^+ on one side of the membrane only, nor the presence of concentrations of Na^+ less than 70 mM on both sides for times up to 30 min (not shown in the Figure) caused an increase in conductance. However, in the presence of Na^+ alone and tryptic digest of *S*, the conductance increased with sodium concentrations as low as 8 mM.

Ionophoric Properties of Both the Large and Small Polypeptides of $(Na^+ + K^+)$-ATPase Together

The next set of experiments was constructed to repeat the format of Figs. 3 and 4 except that the ionophoric material added contained *L* and *S* together in a weight ratio of 1:1. The concentration of each polypeptide was 5×10^{-4}. Fig. 5 is representative of six such experiments. The $t = 0$ re-

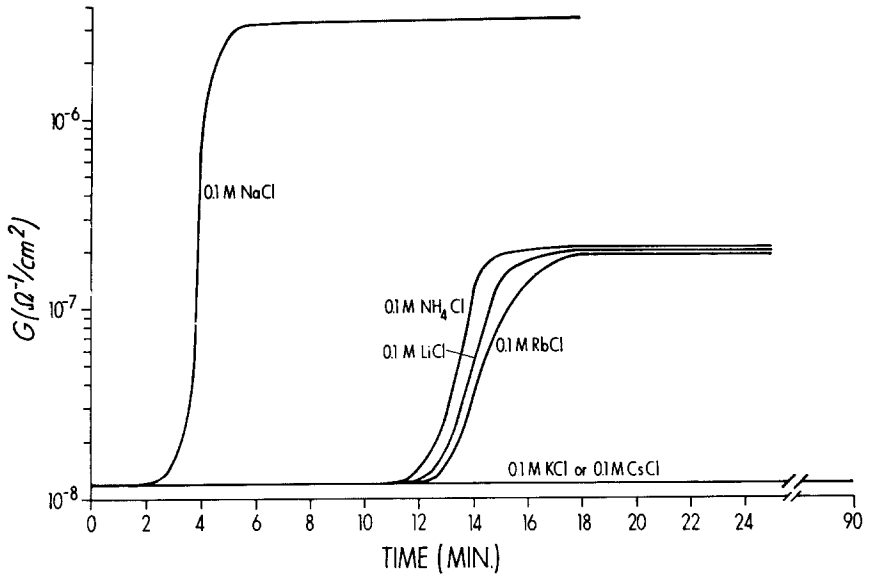

Fig. 5. The time-response of conductance in the presence of $\sim 5 \times 10^{-4}$ mg/ml of the large and small polypeptides of $(Na^+ + K^+)$-ATPase together on a black lipid membrane in the presence of various ions. The membrane was formed, reached a steady state, then the polypeptides were added at $t=0$ into the fluid inside the Teflon cup. The bilayer bathing fluid consists of 0.1 M salt (either NaCl, KCl, CsCl, RbCl, LiCl or NH_4Cl) as indicated, 5 mM histidine, pH 7.4, 10 °C. The conductance of the bilayer was continuously monitored under the conditions of -50 mV voltage clamp. The fluid inside the Teflon cup, which contained the polypeptides, was subjected to a negative voltage with respect to the outside chamber

presents the time of addition of pre-mixed L and S into the bathing fluid of an already-formed bilayer. The original electrolyte solution was 0.1 M salt (NaCl, KCl, etc.), 5 mM histidine, pH 7.4, 19° C. The conductance was continuously monitored under -50 mV voltage clamp.

The presence of Na^+ as compared to the presence of each of the other monovalent cations used in these experiments resulted in dramatic differences in conductance in terms of both the time required to reach maximal increase in conductance and final steady-state conductances. In the presence of K^+ and Cs^+, no increase in conductance was ever observed up to 90 min in at least five experiments with each cation. In the presence of NH_4^+, Li^+ or Rb^+ there was one order of magnitude change in conductance at a much later time than in the presence of Na^+. If the dynamic parameter of time required to reach the maximal increase in conductance and the actual maximal conductances (i.e., steady-state) are used to define specificity but not for bilayer selective permeability, we could conclude that the presence

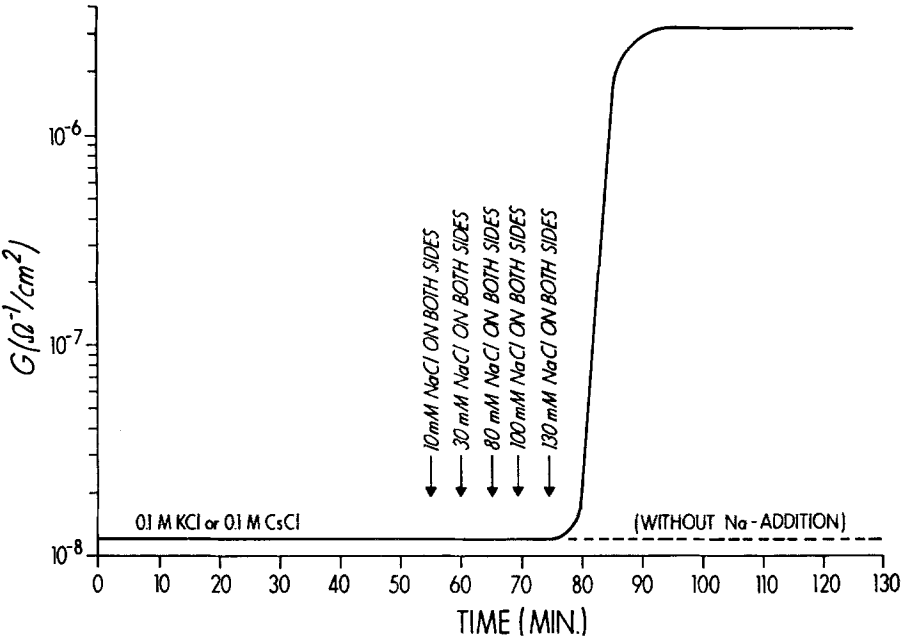

Fig. 6. The effect of Na^+ addition on the time-response of conductance in the presence of $\sim 5 \times 10^{-4}$ mg/ml of both large and small polypeptides of ($Na^+ + K^+$)-ATPase together on a black lipid membrane conductance. The rest of the ionic and voltage conditions are the same as those described for Fig. 5

of both *L* and *S* provided a dramatic specifity for Na^+. The Na^+ requirement in the presence of *L* and *S* together was more marked than the Na^+ requirement in the presence of the tryptic digest of the small peptide (*see* Fig. 5). In the presence of Na^+, K^+ and Cs^+ the variations from one experiment to another were of the order of $\pm 20\%$ in the maximal conductance and the time required to reach a maximum conductance. However, in the case of NH_4^+, Li^+ and Rb^+ the variation from one experiment to another was about ± 20 to 50% for the maximal conductances and the time required to reach such maximal conductances.

The next experiment was designed to illustrate the effect of Na^+ on bilayer conductance in the presence of both *L* and *S* polypeptides in the presence of K^+ or Cs^+. The $t = 0$ in Fig. 6 represents the time of addition of 5×10^{-4} mg/ml of both *L* and *S* into an already-formed bilayer. The original electrolyte bathing solutions were 0.1 M KCl or CsCl and 5 mM histidine, pH 7.4. No increase in conductance was observed for up to 55 min. Neither the addition of Na^+ on one side only nor Na^+ concentrations below 100 mM for times up to 30 min (not shown) caused an increase in bilayer conductance. However, at $Na^+ > 100$ mM on both sides we observed

a large increase in conductance as compared to the bilayer conductance in the absence of Na^+. The requirement for $Na^+ > 100$ mM to increase the conductance in the presence of 0.1 mM KCl was similar to our recent results for the effect of tryptic digest of eel electroplax microsomal $(Na^+ + K^+)$-ATPase on bilayers (Shamoo & Albers, 1973*a*). However, in the presence of Na^+ alone and both polypeptides, conductance increased with concentrations of Na^+ as low as 8 mM.

Molar Ratio of Small-to-Large Polypeptides and its Ionophoric Importance

The next experiment was designed to test the significance, if any, of the weight ratio of 1:1 of large-to-small polypeptides. In a pre-mixed 1:1

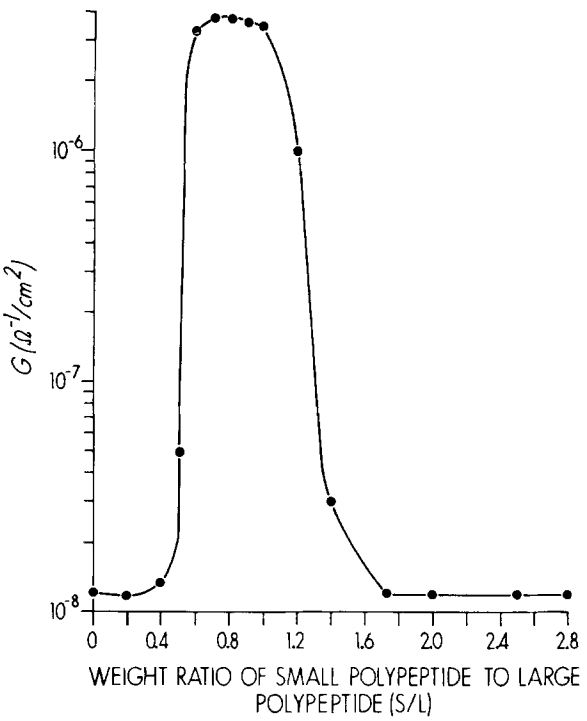

Fig. 7. The effect of varying the pre-mixed weight ratio of small-to-large polypeptide of $(Na^+ + K^+)$-ATPase on black lipid membrane conductance. The bathing fluid consisted of 0.1 M NaCl, 5 mM histidine, pH 7.4, and the two polypeptides in the indicated weight ratios. The large polypeptide was fixed at $\sim 5 \times 10^{-4}$ mg/ml and the small polypeptide was varied. In a different set of experiments, the small polypeptide was fixed and the large polypeptide was varied. The results were similar to the data shown in this figure. The conductance was monitored under -50 mV voltage clamp. Each point represents a separate experiment for the steady-state conductance (> 10 min) reached in the presence of the indicated amounts of the two polypeptides

weight ratio we observed a marked requirement for Na^+ in order to increase the bilayer conductance. Fig. 7 is representative of five experiments on conductance versus the weight ratio of small-to-large polypeptides. The amount of large polypeptide was held constant at 5×10^{-4} mg/ml and the amount of small polypeptide was varied. Similar data were obtained by fixing the amount of small polypeptide constant at 5×10^{-4} mg/ml and varying the amount of the large polypeptide. At weight ratios of small-to-large polypeptides ranging from 0.6 to 1.2 there was a large increase in the Na^+-specific conductance in black lipid membrane. The known figures for molecular weights average about 50,000 for the small polypeptide and about 100,000 for the large polypeptide (Uesugi *et al.*, 1969; Kyte, 1971*a*, 1972; Collins & Albers, 1972; Hokin *et al.*, 1973). Thus, the weight ratio of 1:1 of small-to-large polypeptides may indicate a molar ratio of two small polypeptides to one large polypeptide. Such a triad might be the structure in which the Na^+-specific ionophoric site is exposed. It may also imply that the native enzyme exists in a molar ratio of two small to one large polypeptide. But such a triad formation may be required to demonstrate Na^+-dependent ionophore only in a synthetic bilayer system and not in an *in vivo* system. A molar ratio of two small to one large polypeptide has been suggested by Kyte (1972) for the canine renal medulla $(Na^+ + K^+)$-ATPase. The presence of an excess of one polypeptide to another may cause formation of aggregates larger than triads, thus masking the ionophoric activity as indicated by the increase in bilayer conductance.

Discussion

The increase in BLM conductance under positive voltage (positive on the side of the protein) always took place in the presence of each of the materials used in the report (i.e., large polypeptide, small polypeptide, the two polypeptides together, or the tryptic digest of either large or small polypeptide) and in the presence of any of the monovalent cationic conditions. But under no voltage or negative voltage clamp did the BLM conductance increase with either the tryptic digest of the small polypeptide or the polypeptides together and in the presence of sodium ion. Under the conditions of positive voltage clamp, the data suggest the following possibilities: 1) that a nonspecific ionophore exists which is distinct from the Na^+-dependent ionophore; 2) that neither of the polypeptides is "pure" and the contaminant polypeptides or their tryptic digests have nonspecific ionophoric properties; 3) that the Na^+-dependent ionophore may be

attached to a positively charged peptide which possesses nonspecific ionophoric properties (as measured by the bilayer conductance; 4) that the SDS-ionophore complex (even though dialyzed for several days) may endow the ionophore with nonspecific properties; or 5) particles from the anion exchange resin outside the dialysis chamber may have contaminated the polypeptides. To resolve the possibilities we have to await the further isolation and purification of the different fragments of $(Na^+ + K^+)$-ATPase to assure that no separate protein band(s) is (are) closely associated with the two polypeptides. We might expect that further purification of the two polypeptides would lead to a greater[2] dependence of the increased conductance on the presence of Na^+.

The tryptic digest of the small polypeptide showed a decrease in the Na^+ required to increase the bilayer conductance compared with the Na^+ requirement of the two polypeptides together. One explanation for such an apparent discrepancy is that the tryptic digest might have partially destroyed the specificity of the Na^+-site, whereas when the small and large polypeptides are present together they form a more "native" structure with less destruction of the Na^+-specific site.

The large polypeptide is known to form a high energy phosphorylated intermediate from γ-^{32}P-ATP in the presence of Na^+. There is no evidence that the small polypeptide has any enzymatic function in $(Na^+ + K^+)$-ATPase, but it has been universally associated with the "purified" $(Na^+ + K^+)$-ATPase. The small polypeptide is also known to be a glycoprotein and is not phosphorylated by γ-^{32}P-ATP (Uesugi *et al.*, 1969; Kyte, 1971*a*, *b*, 1972; Collins & Albers, 1972; Hokin *et al.*, 1973). Our data suggest for the first time that the Na^+-specific site (or "Na-carrier") is separate from the main large polypeptide chain which contains the high energy Na^+-induced phosphoprotein.

The order of cationic requirement for the increase in the BLM conductance does not follow the so-called selectivity isotherms (Eisenman, 1962, 1965; Diamond & Wright, 1969) in either the case of both polypeptides together or the tryptic digest of the small polypeptide. With respect to the time required to reach maximal conductance or the maximally reached conductance, NH_4^+ was almost as effective as Na^+ in increasing BLM conductance in the presence of the tryptic digest of the small polypeptide. For a passive system, which is quite different from those we have reported, the similar effectiveness of NH_4^+ to that of Na^+ has been reported for the Na^+-

2 Data not shown on biionic potentials indicate that once the conductance of BLM has increased in the presence of both polypeptides or the tryptic digest of *S*, the bilayer showed no selectivity among the monovalent cations tested.

channel of the sciatic nerve fiber (Hille, 1971) and some derivatives of NH_4^+ ion were reported to partially replace Na^+ in bilayers in the presence of carriers (Eisenman & Krasne, 1973).

It is of interest to note that the sharpest contrast of the effectiveness of various ions in increasing the bilayer conductance in the presence of either the tryptic digest of *S* or both *L* and *S* together was between Na^+ and K^+. K^+-ion failed completely (in up to 2-hr-long experiments) to increase the bilayer conductance in two conditions mentioned above. This is the most significant selectivity in the cell membrane, since Na^+ and K^+ are the major monovalent cations of the fluid bathing the cell membrane.

Further work is needed in several areas: the purification of the Na^+-specific inophore, the elucidation of the role of both Na^+ and K^+ together on the ionophore, and the transduction of energy from the hydrolysis of ATP by ($Na^+ + K^+$)-ATPase via "Na^+-carrier complex."

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