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Interactions of Folch-Lees Proteolipid Apoprotein with Planar Lipid Bilayers

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Summary. Water-soluble Folch-Lees proteolipid apoprotein from bovine CNS white matter induces a voltage-dependent conductance in black lipid membranes. Na^+ is required for the induced conductance change but the established conductance has very low ionic selectivity. The induced conductance fluctuates with a minimum amplitude of $10^{-11}-10^{-10}$ mho. The magnitude of the conductivity change is dependent on protein concentration and on the composition of lipid bilayers. At a fixed voltage the induced conductance of a phosphatidylcholine-cholesterol membrane is proportional to the sixth power of the protein concentration and the first power of Na^+ concentration. The interactions between the apoprotein and the lipids are both electrostatic and hydrophobic, but the interaction leading to the conductance increase appears to be mainly hydrophobic. Both the increase in conductance and the current fluctuations remain after extensive washing of the chambers to remove the protein. Furthermore, pronase or glutaraldehyde added to either the cis or trans side of the membrane does not affect the apoprotein-established conductance. However, if the bilayer is formed in the presence of both the apoprotein and pronase or if the apoprotein is treated with pronase prior to its addition to the chamber, no conductance change is observed. The association of the apoprotein with the membrane thus appears to render the protein inaccessible to proteolytic digestion, suggesting that the apoprotein is at least partially imbedded in the membrane interior.

Model membranes containing well defined proteins and lipids provide a simplified system for the study of the role of lipid-protein interactions in the molecular architecture and function of biological membranes. The major interactions between soluble or extrinsic membrane proteins and lipids are electrostatic, and changes in the membrane conductance in the lipid bilayers are generally not observed (Tien & Mountz, 1976). Integral or intrinsic proteins show both electrostatic and hydrophobic interactions with lipids, but these have been studied to a much lesser extent.

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The Folch-Lees proteolipid, one of the two major proteins of central nervous system myelin, accounts for approximately half of the total myelin protein; most of the remaining protein is the myelin basic protein (Folch-Pi & Stoffyn, 1972). The proteolipid is an amphipathic, intrinsic membrane protein which is soluble in organic solvents and associates closely with lipids. The apoprotein, devoid of complex lipids, maintains its solubility in chloroform-methanol but can be converted to a water soluble form (Tenenbaum & Folch-Pi, 1966; Folch-Pi & Stoffyn, 1972; Hendrickson, Joffe & Davidson, 1972). The interactions of the apoprotein with different lipids have been studied in monolayers (Ter Minassian-Saraga *et al.*, 1973; London *et al.*, 1974) and in liposomes (Braun & Radin, 1969; Papahadjopoulos, Vail & Moscarello, 1975; Boggs, Vail & Moscarello, 1976; Boggs *et al.*, 1977; Curatolo *et al.*, 1977; Cockle *et al.*, 1978*a, b*), and interactions with both charged and neutral lipids have been demonstrated. In the present paper, we describe the effects of the water-soluble form of the proteolipid apoprotein on the electrical properties of bilayers of various lipid compositions.

Materials and Methods

Chromatographically pure bovine phosphatidyl serine (PS), egg yolk phosphatidyl choline (PC), and cholesterol were purchased from Supelco Inc. (Bellefonte, Pa.) and *n*-decane was obtained from Eastman Kodak Co. (Rochester, N.Y.). Pronase was obtained from Sigma Chemical Co. (St. Louis, Mo.) and glutaraldehyde from Taab Laboratories (Emmer Green, Reading, England). All other chemicals were reagent grade.

Bimolecular or black lipid membranes (BLMs) were formed from PS (10 mg/ml *n*-decane) or from PC (20 mg/ml *n*-decane) or a mixture of PC and cholesterol at a concentration of 12.5 mg PC and 3 mg cholesterol per ml *n*-decane. BLMs formed from PC alone were unstable in the presence of the apoprotein. The bilayers were formed by spreading the lipid solution with a brush or a Pasteur pipette across the hole ($\sim 3 \times 10^{-2}$ cm^2) on a polyethylene or Teflon partition, which separates two aqueous phases (Mueller *et al.*, 1964). The protein was added to either one or both chambers after the membrane had thinned down to a black film. The protein-induced conductance usually reached a steady state within 10 min after the addition of the protein to the aqueous phase. To ensure that the newly established membrane conductance had reached a stable value, all current-voltage (*I-V*) curves were taken 15 min after protein addition. Experiments were also performed in which the chloroform/methanol (2:1 vol/vol) soluble form of the protein was combined with the lipid solution prior to formation of the membrane. The rate of thinning was considerably slower when the membrane was formed in the presence of the protein. All aqueous solutions were buffered with 10 mM Tris-acetate at pH 6.5, and all measurements were performed at room temperature (~ 21 °C). Aqueous solutions were changed by perfusion of the chambers using a pair of mechanically matched coupled syringes.

Membrane conductance (G_m) was determined using a four-electrode system. One pair of electrodes was connected to a high input impedance differential amplifier for measuring membrane potential (V_m). A third electrode was connected to a voltage source (a ramp or a pulse generator), and the fourth was used to measure the current flowing across the membrane (I_m). A potential was defined as positive when the potential of the back chamber was more positive than the front, and a positive current was considered as one in which cations flow from the back to the front chamber. G_m ($G_m = I_m/V_m$) was calculated from the current-voltage curves at a constant voltage. Ag-AgCl electrodes were used for most measurements, but for measurements which involved asymmetric chloride concentrations across the bilayer, commercial calomel electrodes were substituted.

The proteolipid apoprotein was isolated from bovine white matter and delipidated by dialysis against neutral and acidic chloroform-methanol (Lees & Sakura, 1978). After the removal of lipids, the apoprotein was converted to the water-soluble form by the method of Sherman and Folch-Pi (1970). Detergent was not used at any step of the isolation or purification procedures. The apoprotein, thus prepared, is free of complex lipids but still contains 2–4% covalently bound fatty acids (Stoffyn & Folch-Pi, 1971). The protein shows a single, sharp band upon isoelectric focusing in a non-ionic detergent (Draper, Lees & Chan, 1978), but multiple bands are observed on SDS polyacrylamide gels. However, no chemical differences have been detected in the material eluted from the various gel bands and a structural interrelationship among the bands has been proposed (Lees *et al.*, 1979). Bovine myelin basic protein was kindly provided by Dr. Eugene Day of Duke University.

Results

Symmetrical Addition of Apoprotein

The addition of the proteolipid to both sides of the membrane separating two aqueous solutions containing 0.1 M buffered NaCl induces an increase in membrane conductance (Fig. 1). No difference is observed between results obtained when protein is added to the aqueous chamber after formation of the lipid membrane or when the chloroform-methanol soluble protein is mixed with the lipid solution prior to membrane formation. Results are shown only for the former set of experiments. The zero-voltage conductance of the PC-cholesterol membranes is $\sim 10^{-7}\ \Omega^{-1}\cdot cm^{-2}\cdot \mu g^{-1}$ protein, whereas that of the PS membranes is an order of magnitude lower ($\sim 10^{-8}\ \Omega^{-1}\cdot cm^{-2}\cdot \mu g^{-1}$ protein). Regardless of the lipid composition of the membrane, the current increases exponentially with the voltage. For each 25 mV change in potential, there is an *e*-fold change in membrane current (insert of Fig. 1). In addition to the voltage dependent increase in membrane conductance, the apoprotein induces random current fluctuations at voltages greater than ± 20 mV. These fluctuations occur only after the increase in membrane conductance is observed and have a minimum amplitude of 10^{-11}

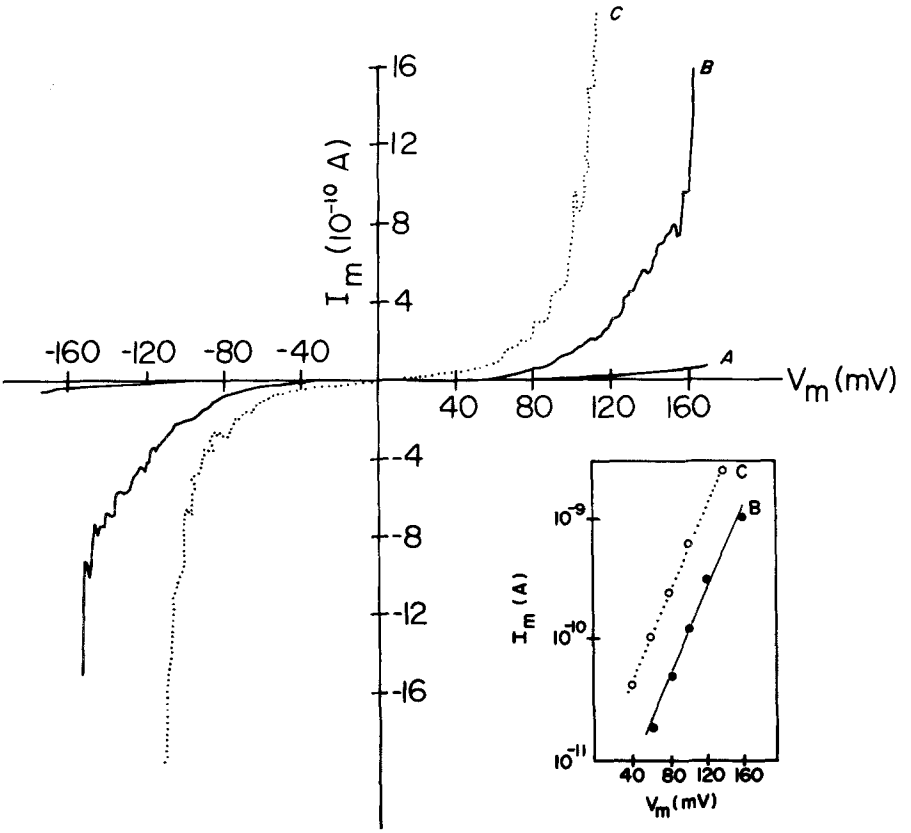

Fig. 1. Current-voltage relationship for (*A*) PS or PC-cholesterol BLM with no Folch-Lees proteolipid apoproten present in the aqueous phase. PC-cholesterol BLM without apoprotein has the same membrane conductance as that of the PS control BLM; *(B)* PS BLM and 2.5 μg/ml apoprotein; *(C)* PC-cholesterol BLM and 1.0 μg/ml apoprotein. Membranes were formed in 0.1 M NaCl and 0.01 M Tris-acetate buffer, pH 6.5, and the protein was added to both chambers. Insert is a replot of curve *A* on a semi-logarithmic scale to show the exponential dependence of the current on voltage

to 10^{-10} mho. On the basis of preliminary single-channel analyses, the amplitude of the fluctuations appear to be independent of the applied voltage between ± 20 and ± 90 mV. Both the increase in conductance and the current fluctuations remain after extensive washing of the chambers to remove the protein, suggesting that the protein either penetrates the lipid bilayer or is irreversibly adsorbed to it.

Asymmetrical Addition of Apoprotein

Asymmetry in the I-V curves is observed if the protein is added to only one side of the bilayer (Fig. 2). The positive current flows more

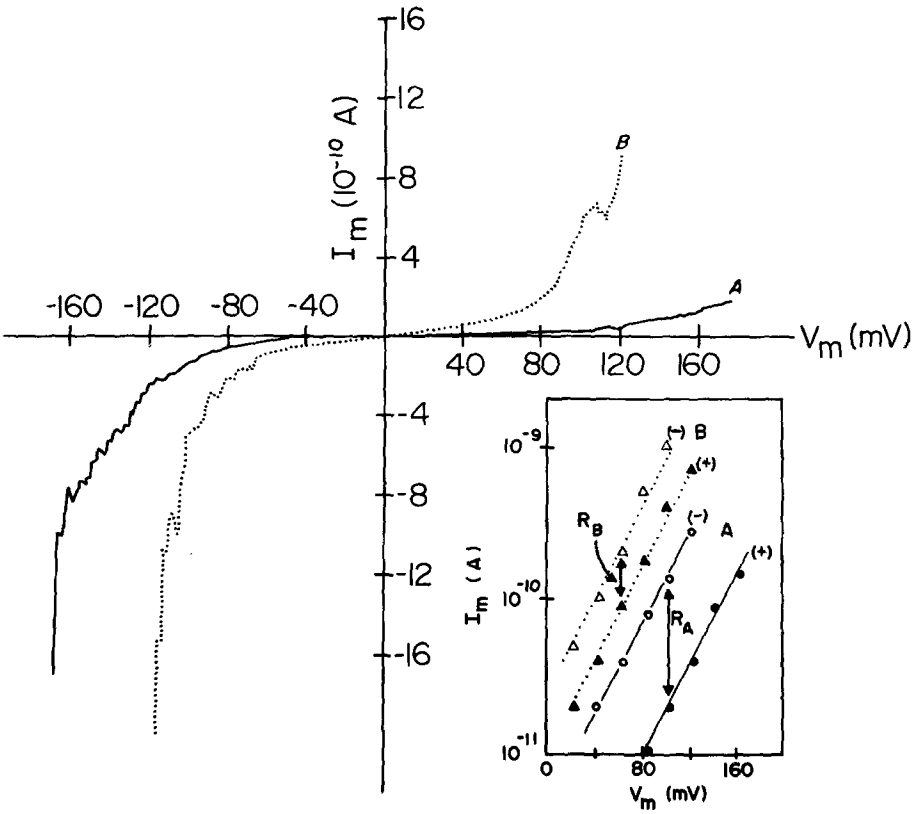

Fig. 2. Current-voltage relationship for (*A*) PS BLM and 2.5 μg/ml apoprotein; (*B*) PC-cholesterol BLM and 1.0 μg/ml apoprotein. Experimental conditions are the same as in Fig. 1 except the protein was added to the front chamber only. Insert is the replot of the *I-V* curves on a semi-logarithmic scale. Closed symbols are for positive voltages and the open symbols are for negative voltages

easily away from the side containing the protein than towards it. However, the asymmetry is less pronounced in the PC-cholesterol membrane than in the PS membrane. The magnitude of the asymmetry is defined as the distance (R) between the two curves of the positive and negative voltages on a semi-log plot of current *vs.* voltage (insert of Fig. 2). The value of R for the PC-cholesterol bilayer is approximately half that of the PS bilayer.

Membrane Stability with Apoprotein Incorporated

Both PS and PC-cholesterol BLMs are normally stable for several hours, even after incorporation of apoprotein. The modified bilayers

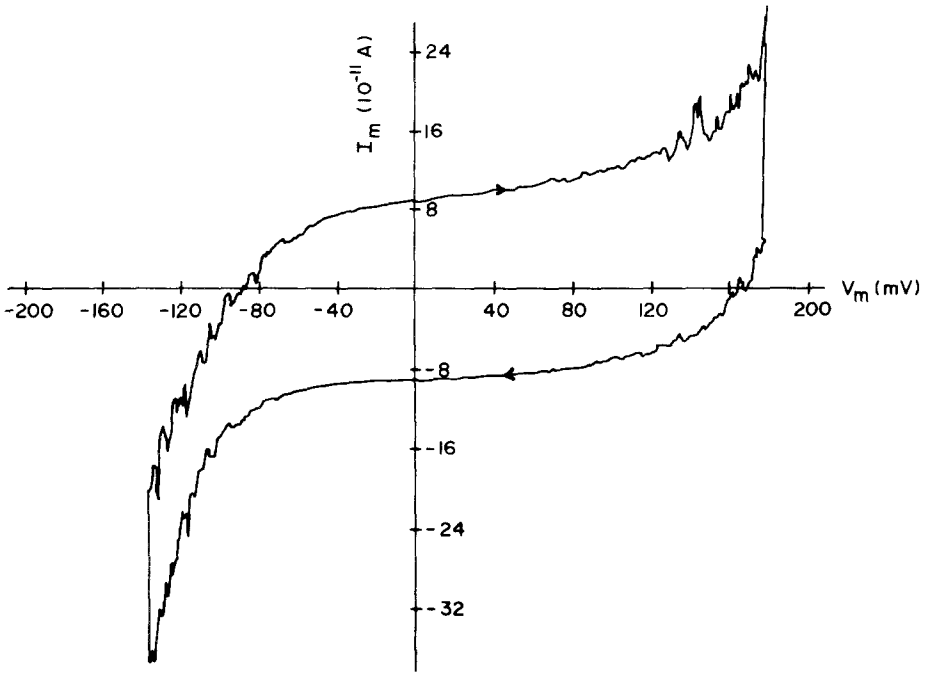

Fig. 3. Current-voltage curve of a PS BLM with addition of 2.5 μg/ml apoprotein to the front chamber. Experimental conditions are the same as in Fig. 1. Arrows show direction of voltage sweep; $dv/dt = 10$ mV/sec

can be subjected to repeated voltage sweeps up to ± 150 mV without showing any sign of breakage or increase in membrane conductance above the level of the apoprotein-induced membrane conductance. Figure 3 shows a typical *I-V* curve of a PS-apoprotein bilayer measured at a voltage sweep rate of 10 mV/sec. The separation of the traces in the two different sweep directions (indicated by arrows) is due to the capacitive charging current (I_c) and is given by $I_c = C(dv/dt)$ where C is the membrane capacitance, and dV/dt is the change of membrane voltage with time. In contrast to these results, the PC-apoprotein recombinent membranes are relatively unstable under these conditions and reproducible measurements cannot be obtained.

Ion Selectivity

Sodium ions (> 1.0 mM) on the same side of the membrane as the apoprotein are required for the protein to exert an effect on the bilayer,

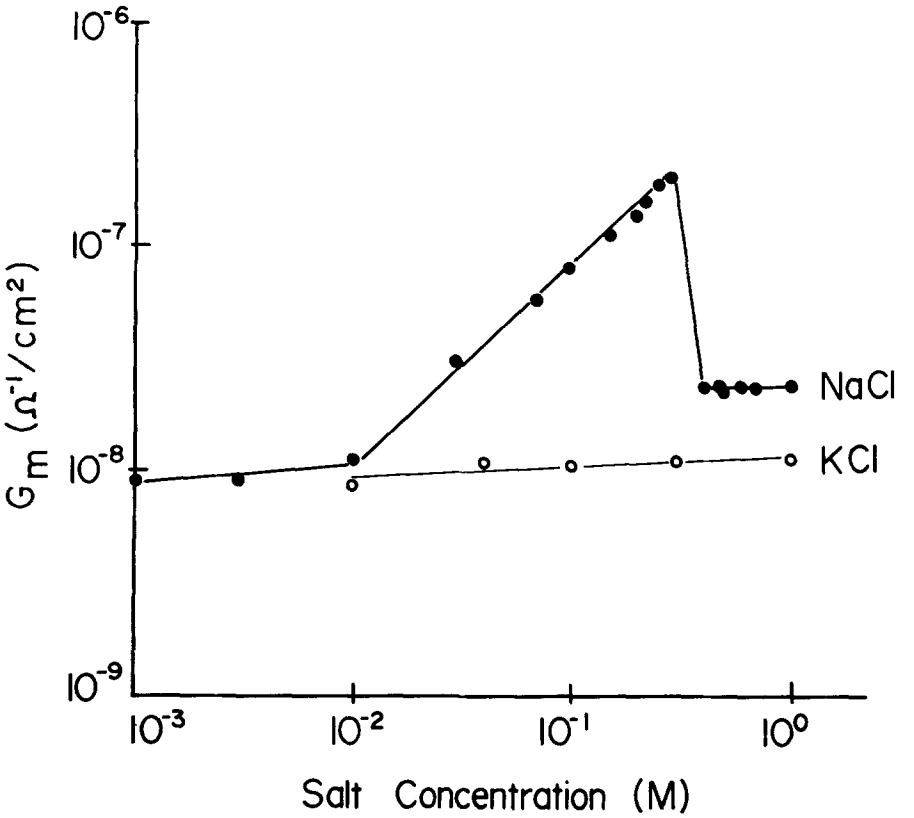

Fig. 4. Effect of either NaCl or KCl on membrane conductance (V_m=60 mV) of PC-cholesterol BLM at a fixed concentration of the apoprotein (1.0 μg/ml) in both chambers. All aqueous solutions were buffered with 0.01 M Tris-acetate at pH 6.5

but, once the increase in conductance has been produced, the membrane loses its selectivity for Na^+ and K^+. The bionic potential, i.e., the electrical potential difference at zero current across a membrane separating equimolar solutions of NaCl and KCl, is zero. Measurements of zero current potential in the presence of a ten-fold gradient of NaCl concentration indicates that the selectivity for cations and anions is insignificant: the transference number for Na^+ is only 0.4. However, if only KCl is present, the apoprotein has no effect on the conductance (Fig. 4). In contrast, at a fixed protein concentration, the effect of the apoprotein on the membrane conductance is directly proportional to NaCl concentration in the range between 0.01 and 0.3 M (Fig. 4). The conductance reaches a maximum at around 0.3 M NaCl and drops precipitously beyond this concentration almost to the level of the unmodified membrane. The deviation from linearity in the data at low NaCl concentra-

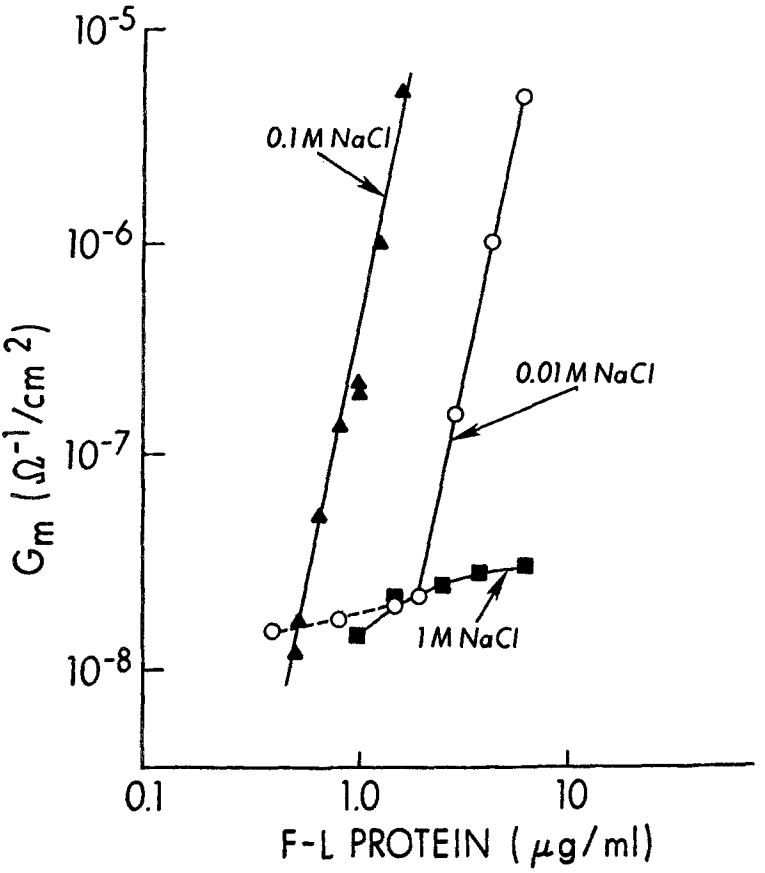

Fig. 5. Effect of Folch-Lees proteolipid apoprotein concentrations on membrane conductance (V_m=60 mV) of PC-cholesterol BLM at three different NaCl concentrations. All aqueous solutions were buffered with 0.01 M Tris-acetate at pH 6.5. Apoprotein was present in both chambers

tions is probably due to the relatively small magnitude of the changes in conductance induced by the protein at the low salt concentration.

Apoprotein Concentration

With the apoprotein in both chambers, the conductance of a PC-cholesterol BLM in either 0.01 or 0.1 M NaCl increases as the sixth power of the protein concentration (Fig. 5). Unilateral addition of protein gives the same slope, but the curve is shifted to the right along the protein concentration axis. At high salt concentration (1.0 M NaCl) the addition of protein causes only minimal change in conductance. Further

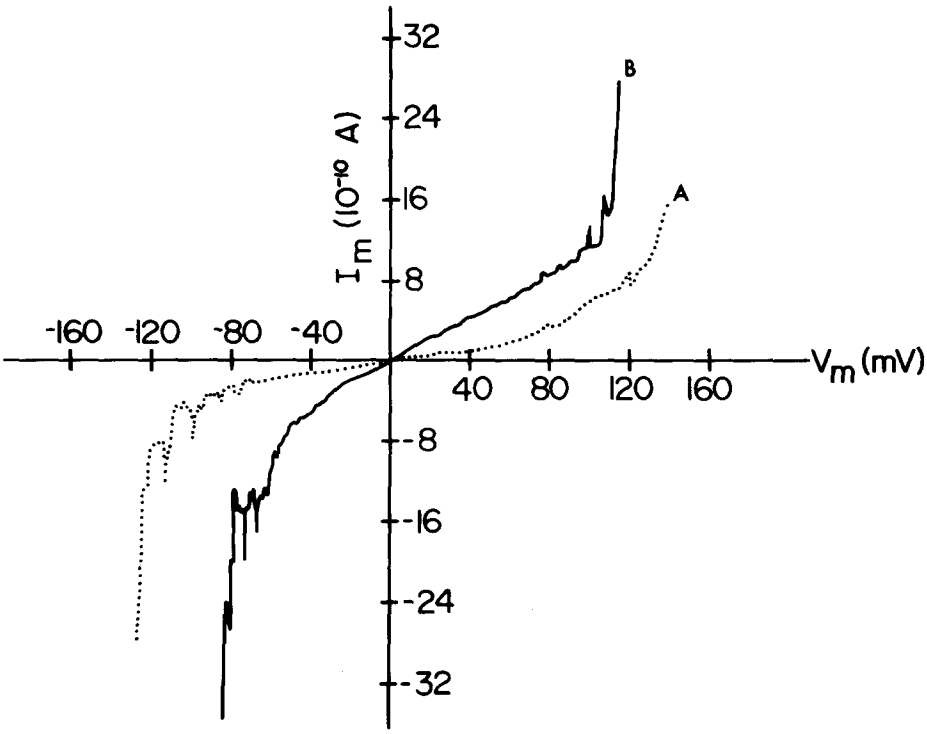

Fig. 6. Current-voltage curves of a PS bilayer exposed to 3.3 μg/ml apoprotein in the front chamber (*A*), and subsequent addition of 10 μg/ml myelin basic protein to the back chamber (*B*). Membrane was formed in 0.1 M NaCl and 0.01 M Tris-acetate buffer, pH 6.5

increase in protein concentration produces little additional conductance change.

Other Myelin Proteins

In contrast to the effect of the proteolipid apoprotein on the electrical proteins of a PS bilayer, the addition of myelin basic protein at concentrations up to 10 mg/ml has no effect. However, upon addition of basic protein after establishment of the conductance increase by the apoprotein, a further enhancement of the membrane conductance is observed (Fig. 6) and the membrane becomes unstable.

Apoprotein Modifications

The increase in membrane conductane is not observed after the protein has been subjected to prolonged freezing and thawing. Further,

if the apoprotein is incubated for 30 min with pronase (50 μg/ml) prior to its addition to the chamber, no increase in conductance is observed. Similarly, if the bilayer is formed in the presence of both the apoprotein and pronase, no conductance change is observed. In contrast, if the apoprotein is added to the bilayer and pronase is subsequently added to either the same or the opposite side, the apoprotein-induced increase in conductance and the amplitude of the current fluctuations are maintained. Similarly, the addition of up to 2% glutaraldehyde to the aqueous medium has no effect. Thus, once the apoprotein has interacted with the BLM, it appears to be inaccessible to proteolytic digestion or chemical fixation.

Discussion

The results presented in this paper demonstrate that the bovine brain white matter proteolipid apoprotein, when incorporated into a BLM, increases membrane conductance and that Na^+ is required to produce this change. It is well established that the apoprotein shows a conformational flexibility in different solvents (Sherman & Folch-Pi, 1970; Moscarello, *et al.*, 1973). Salts are known to alter the interrelationship between this protein and lipids and to cause aggregation of the protein (Webster & Folch, 1961). It is, therefore, possible that a specific conformational change in the protein may occur in the presence of Na^+ and that it is only in this conformation that the protein can interact with the BLM. Although no differences have as yet been observed between the effects of different monovalent cations on the conformation of the protein, the occurrence of such changes is not precluded. At high Na^+ concentrations (> 0.3 M) a nonspecific aggregation occurs (Butler, 1975). The large complexes which are formed may not be able to penetrate the bilayer, and consequently little change in membrane conductance is observed (Figs. 4 and 5).

The magnitude of the apoprotein-induced membrane conductance change is dependent on the composition of the BLM and reflects a difference between electrostatic and hydrophobic forces. In the case of cholesterol-containing membranes, hydrophobic interactions probably account for the marked effect of the apoprotein on the PC-cholesterol BLM. The increase in conductance observed with increasing ionic strength (Fig. 4 and 5), along with the evidence from the monolayer and liposome studies (Braun & Radin, 1969; London *et al.*, 1974; Papa-

hadjopoulos *et al.*, 1975; Boggs *et al.*, 1976; 1977; Cockle *et al.*, 1978*a*, *b*), all emphasize the importance of hydrophobic interactions between the apoprotein and the lipids. On the other hand, with a comparable amount of protein, the conductance change observed for a PS BLM is less than for the PC-cholesterol BLM. The apoprotein bears a net positive charge at pH 6.5 (isoelectric point = 9.2, Draper, Lees & Chan, 1978), and the positively charged protein molecules can be expected to interact with the negative charges of the PS head groups. Consequently, fewer protein molecules would be available for hydrophobic interactions and a lesser magnitude of the conductance change would be expected.

Both proteolipid apoprotein and myelin basic protein have been shown to interact with charged lipids at an air-water interface and to expand the area of the monolayer at a constant surface pressure (Demel *et al.*, 1973; London *et al.*, 1974). However, in the present study the myelin basic protein produced no change in membrane conductance when added to either one or both sides of a PS BLM. Myelin basic protein is a water soluble, hydrophilic protein which probably interacts with membranes mainly via electrostatic forces. Soluble basic proteins generally interact electrostatically with charged lipids and can thereby alter the permeability of PS vesicles to $^{22}Na^+$ (Kimelberg & Papahadjopoulos, 1971*a*). However, the extent of the effect of different basic proteins on permeability varies by several orders of magnitude and appears to correlate with the degree of penetration of the protein into PS monolayers (Kimelberg & Paphadjopoulos, 1971 b). Myelin basic protein has been shown to markedly alter the permeability of liposomes to glucose (Gould & London, 1972). This effect has been interpreted as a consequence of the pentration of the lipid matrix by the protein, or certain regions of the protein, resulting in increased permeability of the liposomes to glucose. Under the conditions used in the present study, the myelin basic protein does not alter the conductance of the BLM but does increase the conductance of a membrane in which proteolipid apoprotein is already incorporated. This effect could involve either an interaction of the basic protein with a lipid bilayer whose structure has already been altered by the apoprotein or a direct interaction between the basic protein and the proteolipid apoprotein. Preliminary studies on the primary structure of the apoprotein suggest a segregation of amino-acid residues into charged and uncharged regions (Chan & Lees, 1978). Interactions of hydrophobic regions of the protein with the apolar lipid core of the bilayer may fix the protein in a conformation which permits electrostatic interactions. Thus, the membrane conductance, which in-

creases as a result of incorporation of the apoprotein into the membrane, increases further upon interaction with the basic protein. Although the latter interaction could involve mainly electrostatic forces, the possibility of hydrophobic interactions between the basic protein and proteolipid is not precluded. An analogous situation is seen in the increased conductance of BLM produced by a erythrocyte sialoglycoprotein and the further increase in conductance upon the addition of concanavalin A (Tosteson, Lau & Tosteson, 1973).

In addition to the effects on membrane conductance, the apoprotein, once incorporated into the bilayer, produces random current fluctuations which are independent of the lipid composition of the membrane. These fluctuations suggest the formation of channels in the bilayer. They are not as discrete as would be observed upon formation of stable channels, and they could correspond to detergent-like effects of the amphipathic apoprotein. However, several lines of evidence suggest that the current fluctuations are not a consequence of a breakdown of the membrane but are an indication of channel formation. The membrane remains stable at voltages as high as 150 mV and can be subjected to repeated changes in voltages in either direction without changes in apoprotein-induced conductance. Furthermore, the amplitude of the single channel is independent of applied voltage in the range between 20 and 90 mV. Thus, there is no evidence for membrane breakage under these conditions. The apoprotein, as has been observed for most channel formers (Mueller & Rudin, 1968; Muller & Finkelstein, 1972; Eisenberg, Hall & Mead, 1973; Blumenthal & Shamoo, 1975), produces a high order of concentration-dependent conductance change in BLMs. The slope of the log-log plot of conductance *vs.* apoprotein concentration is 6. This high order of dependence on protein concentration supports the hypothesis that the apoprotein forms channels in the bilayer and that the formation of channels involves some type of cooperativity or aggregation. The possible existence of oligomeric forms of the protein proposed by Chan and Lees (1974) suggests that this type of interaction may play an important role in the process of channel formation by the apoprotein. It is likely, but not yet established, that the increase in steady-state conductance produced in the bilayer by the apoprotein is entirely due to the formation of these channels. Further investigation of this possibility is required.

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