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Protein Interactions with Lipid Bilayers: The Channels of Kidney Plasma Membrane Proteolipids

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Summary. Proteolipids extracted from bovine kidney plasma membrane induce irreversible changes in the electrical properties of lipid bilayers formed from diphytanoyl phosphatidylcholine. The interaction with the proteolipid produces channels which are cation selective. At low protein concentrations (i.e., < 0.6 μg/ml), the single-channel conductance is approximately 10 pS in 100 mM KCl and 3 pS in 100 mM NaCl. In the presence of protein concentrations above 1 μg/ml, another population of channels appears. These channels have a conductance of about 100 pS in 100 mM KCl and 30 pS in 100 mM NaCl. Further, these channels are voltage dependent in KCl, closing when the voltage is clamped at values ≧30 mV. The steady-state membrane conductance, measured at low voltages, was found to increase proportional to a high power (2–3) of the proteolipid concentration present in one of the aqueous phases. In 100 mM NaCl, the conductance increases at protein concentrations above 5 μg/ml, whereas in 100 mM KCl it increases at protein concentrations above 0.6 μg/ml. These measurements indicate that the higher steady-state conductance observed in KCl at a given proteolipid concentration in a multi-channel membrane presumably results because more channels incorporate in the presence of KCl than in the presence of NaCl.

The two major fractions which comprise the proteolipid complex were also tested on bilayers. It was found that both fractions are required to produce the effects described.

Key words: Proteolipids, lipid bilayers, protein-induced channels

Proteolipids are ubiquitous components of membranes in animals, plants and microorganisms [6]. These hydrophobic proteins are operationally defined by their solubility in chloroform/methanol. They are further characterized by the amphipatic nature of the apoprotein which is soluble both in water and in chloroform/methanol [5]. In spite of their relative abundance in different membranes, little is known about the structure and properties of proteolipids and their possible role as integral membrane components. In the past years, it has been suggested that the mitochondrial proteolipids might be crucial components of the oligomycin-sensitive ATPase and of the cytochrome oxidase [1–4]. MacLennan and coworkers [14] isolated and characterized a proteolipid from sarcoplasmic reticulum vesicles. This proteolipid was found to copurify with the Ca-ATPase [13] and may play a direct role in the uptake of Ca in the sarcoplasmic reticulum [16]. Forbusch et al. [7] have recently described the identification of a proteolipid in kidney (Na+K)ATPase as a secondary binding site for ouabain. Sapirstein has compared the molecular weight and amino acid composition of kidney plasma membrane proteolipids with the proteolipids obtained from purified preparations of the (Na+K)ATPase from dog kidney and from the shark's rectal gland and found them to be virtually identical. These data suggest that proteolipids may participate in specific membrane functions, perhaps as subunits of membrane transport components. In order to begin to address this question, we set out to learn if the proteolipids from kidney plasma membrane interact with bilayers as measured by changes in the electrical properties of these membranes under various ionic conditions. We have found that the apoproteolipids from kidney plasma membrane interact irreversibly with bilayers made from diphytanoyl phosphatidylcholine. This interaction leads to the formation of channels which are selective for cations and which are voltage dependent at protein concentrations above 1 μg/ml. We have further found that both fractions which comprise the bovine kidney plasma membrane proteolipids are necessary for the effects to occur.

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Materials and Methods

Bilayers were formed from diphytanoyl phosphatidylcholine (12.5 mg/ml pentane; Avanti Biochemicals, Birmingham, Ala.) by apposition of two monolayers spread at the air/solution interface [15]. The hole in the Teflon partition (0.05–0.1 mm^2) was pretreated with a 3% solution of squalene (Eastman Kodak, Rochester, N.Y.) in pentane (Fisher Scientific, Fairlawn, N.J.). The aqueous solutions were buffered to pH 7.0 with 5 mM Tris-Cl or with phosphate. The contents of both chambers could be stirred continuously with magnetic stirrers. All experiments were conducted at room temperature (approximately 20 °C).

The steady-state membrane conductance (G_m) was determined in the limit of zero applied potential by measuring the steady-state current flowing across the membrane in response to an applied potential difference, using silver-silver chloride electrodes. The amplifier used to measure current is an ultra low bias FET input with 1 MHz bandwidth (42 K, Analog Devices, Norwood, Mass.). Membrane potentials (PD) due to different salts or different salt concentrations on both sides of the bilayer were measured using calomel electrodes or silver-silver chloride electrodes coupled to the solutions with salt bridges. The electrodes were then connected to a high input impedance differential voltage amplifier (AD 523, Analog Devices, Norwood, Mass.), the output of which was connected to a chart recorder. The sign of the transmembrane potential is the sign of the compartment to which the protein is added (*cis* compartment). Positive charge flowing from the *cis* to the *trans* compartment is plotted as positive (upward) current. Whenever reported, complete change of the solution on one or both compartments was accomplished using a pair of matched, mechanically coupled syringes.

Proteolipids were isolated and delipidated as previously described [11]. Briefly, the plasma membrane fraction of bovine kidney was extracted with chloroform/methanol (2:1, vol/vol) and the extract partitioned by the addition of water. The final volume ratio of chloroform/methanol/water was 8:4:3. The lower phase was concentrated on a rotary evaporator and the sample acidified with 1% acetic and 0.01 N HCl prior to chromatography on Sephadex LH-60 (Pharmacia Fine Chemicals, Piscataway, N.J.). The elution of protein was monitored at 280 nm with a Pharmacia UV-1 monitor, and the elution profile is shown in Fig. 1. The proteolipid apoproteins, free of lipids, were converted to their water-soluble form by the procedure of Sherman and Folch-Pi [18]. Acid was removed by dialysis against water. The proteolipid in water (100–250 μg/ml) was kept at 4 °C and used within one month of the conversion to the aqueous form.

Proteolipids were prepared for electrophoresis by two methods: *Method 1:* proteolipids in chloroform/methanol were dried down under nitrogen and solubilized in 1% sodium dodecyl sulfate (SDS). *Method 2:* proteolipid apoproteins were converted to the water soluble form followed by addition of SDS. Electrophoresis of samples (25–50 μg) was carried out on 12 cm polyacrylamide gels (PAGE) (9.25% acrylamide, 0.24% bis-acrylamide in 75 mM Tris-acetate pH 7.9, 0.1% SDS). The purified subunits were analyzed on 15% polyacrylamide gels as described by Laemmli [9]. Electrophoresis was carried out at a constant current of 3 mA per tube.

Sodium dodecyl sulfate (SDS) gel electrophoresis of the bovine plasma membrane proteolipid prepared by Methods *1* and *2* is shown in Fig. 2*A*. Two features of this figure are worth noting: (a) the proteolipid appears as an oligomeric series with a monomeric molecular weight around 12,000 D (lane 1), and (b) there is heterogeneity in the low molecular weight region, with a second major species at 13,000 D. This heterogeneity could be resolved by treating the same proteolipid preparation with Method *2* in order to have only the monomeric species present. Figure 2*A* (lane 2) shows that there are two components in the low molecular weight region with molecular weights of 11,500 and 13,000 D. To obtain the separated components, the fractions labeled *FI* and *FII* in Fig. 1 were collected after elution from Sephadex LH-60. These samples were not subjected to further manipulations, other than conversion to the water-soluble form and removal of acid prior to the test in bilayers. Figure 2*B* (lanes 1 and 2) shows the SDS gel electrophoresis of these components treated by Method *2*.

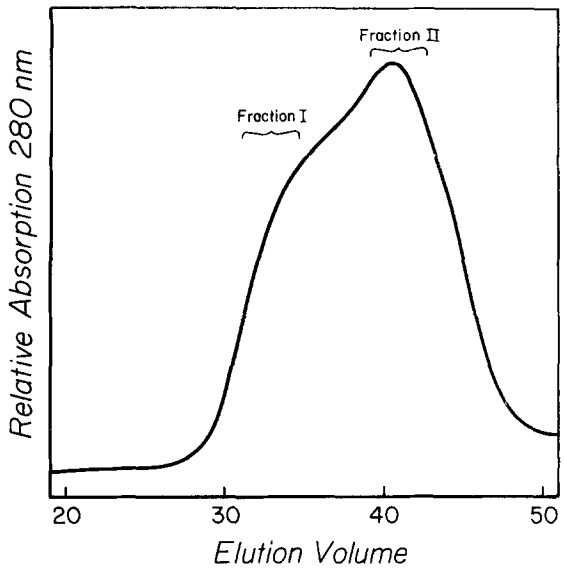

Fig. 1. LH-60 chromatography of bovine kidney plasma membrane proteolipids. Ten mg of protein was loaded on a column (1.5 × 75 cm) equilibrated with chloroform/methanol/acetic acid (2:1:0.03, volume) and monitored continuously at 280 nm. Arrows indicate the fractions taken as fraction *I* and fraction *II*

Results

Addition of apoproteolipids to one of the aqueous phases (*cis*) surrounding a diphytanoyl phosphatidylcholine bilayer induces the formation of channels, as evidenced by discrete current fluctuations seen at constant applied potential. Figure 3 shows the single channels at low protein concentration (0.3–0.6 μg/ml). The single-channel conductance was found to be in the order of 10 pS in 100 mM KCl and around 3 pS in 100 mM NaCl and independent of voltage. When the protein concentration was increased above 1 μg/ml, the current at a fixed potential increased 20 to 30 min after protein addition in one to three steps to its new steady-state value. The step size was found to be about 10 to 30 times the size of the single channels described above. Figure 4 shows that at these higher concentrations of proteolipid, a different channel population becomes evident, both in the presence of NaCl and in the presence of KCl. The conductance of the smallest unit is in the order of 30 pS in 100 mM NaCl and 100 pS in 100 mM KCl. Furthermore, the channels seem to be voltage-dependent and opened at voltages $\leqq 10$ mV, closing when the imposed volt-

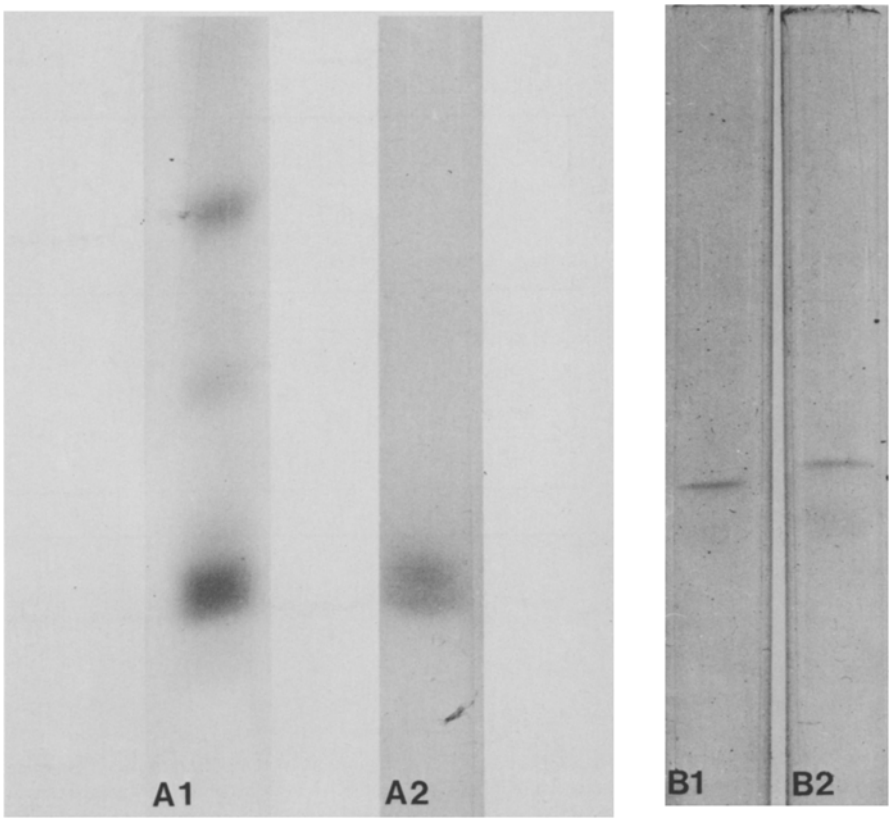

Fig. 2. Gel electrophoresis of bovine kidney plasma membrane proteolipids. Electrophoresis of unfractionated proteolipids prepared by Method 1 (*A*, lane *1*) or Method 2 (*A*, lane *2*) was carried out using Tris-acetate buffer system. Electrophoresis of isolated fraction *I* (*B*, lane *1*) and of isolated fraction *II* (*B*, lane *2*) prepared by method 2 was followed by the procedure of Laemmli [9]

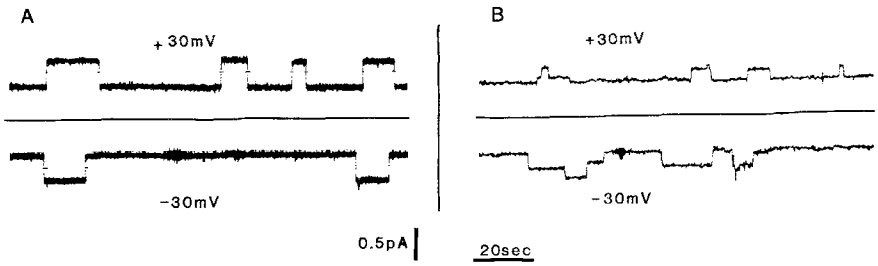

Fig. 3. Single-channel events in membranes treated with a small amount of proteolipid in one compartment. The horizontal lines indicate the zero current level. (*A*): Aqueous solutions: 100 mM KCl, 5 mM PO_4, pH 7.0; proteolipid: 0.3 μg/ml. (*B*) Aqueous solutions: 100 mM NaCl, 5 mM PO_4, pH 7.0; proteolipid: 0.6 μg/ml

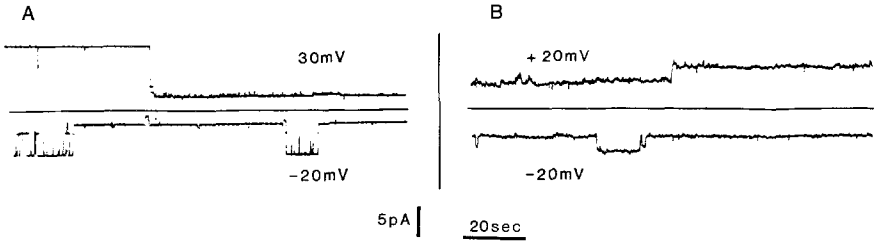

Fig. 4. Time course of membrane current. Same membranes as in Fig. 3, to which more proteolipid was added to a concentration of 1.2 μg/ml (*A*) or 12 μg/ml (*B*)

age is about 30 mV (e.g., Fig. 4*A*). Once the channel closes, it can remain in this state for at least 2 min (not shown). Channels would open again when the absolute value of the applied voltage is zero or less than 30 mV. The bilayer conductance, determined at low voltages ($\leqq 10$ mV) was found to be proportional to the protein concentration. Figure 5 shows that as the protein concentration in one of the aqueous phases is increased, the conductance increases 10-fold for a two- to threefold increase in the proteolipid concentration.

The data presented in Figs. 3–5 strongly suggest

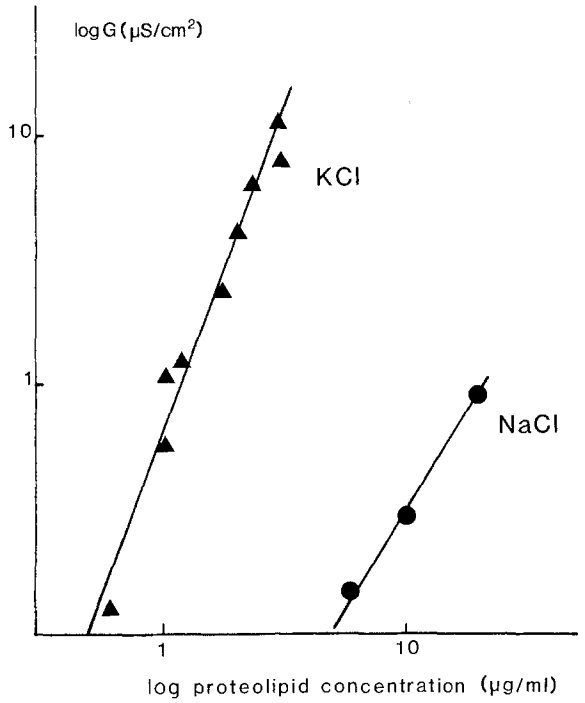

Fig. 5. Double logarithmic plot of membrane conductance *vs.* proteolipid concentration. Membranes were formed in symmetrical salt solutions [100 mM KCl, 5 mM PO_4, pH 7.0 (▲) or 100 mM NaCl, 5 mM PO_4, pH 7.0 (●)]. Successive additions of proteolipids were made to one compartment, and the membrane conductance was measured at steady state. Slope of the lines: KCl, 2.8; NaCl, 1.7

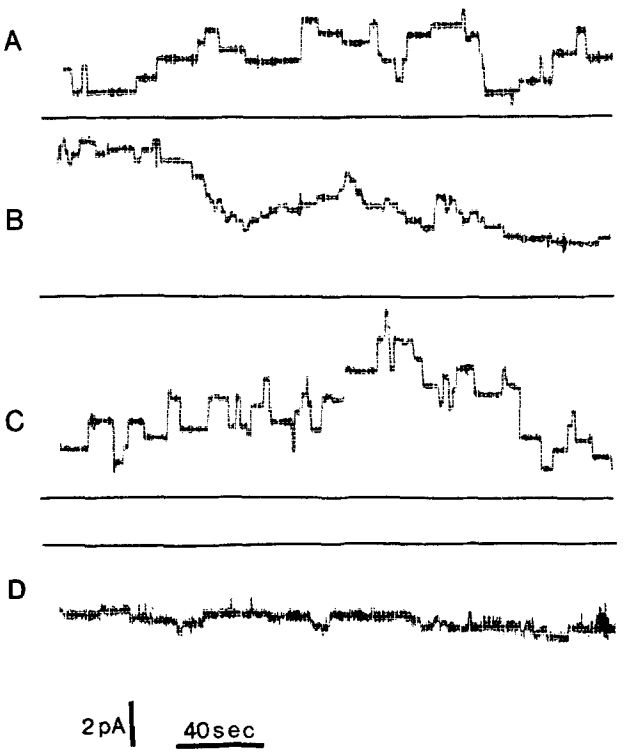

Fig. 6. Time course of membrane current. Bilayer was formed in 5 mM Tris-Cl, pH 7.0, and KCl added to the *cis* compartment to a final concentration of 100 mM. NaCl was added to *trans* compartment to a final concentration of 100 mM. Transmembrane potential (at $I=0$), PD = 0. Proteolipid (1.0 μg/ml) was added to *cis* compartment. After increase in membrane conductance, PD = −18 mV. Mean value of PD was found to be −23 mV (range: −18 to −26 mV). This corresponds to a permeability ratio, $P_K/P_{Na}=2.5$ using $PD=(RT/F)\ln(P_{Na}/P_K)$ [19]. Records *A* through *C*, (V–PD) clamped at +20 mV (V is the imposed potential). Record *D*, (V–PD) = −20 mV. Records *A*, *C* and *D*: 100 mM KCl in *cis* compartment, 100 mM NaCl in *trans* compartment. Record *B*: 0.1 mM NaCl in *cis* and *trans* compartments. For experimental details, *see* text. Solid lines correspond to zero current

that the mechanism by which the proteolipid induces an increase in the bilayer conductance is via channel formation. The simplest interpretation of the high power dependence of conductance on concentration is that the channel is formed by an aggregate of proteolipid molecules. Examples of channels made from oligomers abound in the literature [8, 10].

From the data shown in Fig. 5, it is seen that the steady-state conductance when KCl (100 mM) is the major electrolyte is 100 to 1,000 times higher than when NaCl (100 mM) is the major electrolyte. On the other hand, the ratio of the single-channel conductance in KCl to that in NaCl is about three (e.g., Figs. 3 and 4). These results could be explained if the conformation of the apoproteolipid was different and less favorable to membrane incorporation in the NaCl than in the KCl solution. Thus, for a given protein concentration in the aqueous phase, there would be less protein in the bilayer when NaCl rather than KCl is the salt in the aqueous phase. This would lead to fewer channels and hence to lower steady-state membrane conductance. Once in the bilayer, though, the proteolipid would assume the same conformation so that the permeability to K^+ would be three times that for Na^+ *independent* of the cation present at the time of incorporation. The steady-state conductance, on the other hand, would depend on the cation present on the side where the protein is added.

Figure 6 shows the results of experiments designed to test this hypothesis. After the membrane was formed in 5 mM Tris-Cl, enough 3 M KCl was added to the *cis* compartment to have 100 mM KCl as the final concentration. NaCl was added to the *trans* compartment, its final concentration also being 100 mM. The apoproteolipid was added to the *cis* compartment, and Fig. 6*A* shows the current fluctuations observed when the driving force for current flow through the channel (V–PD) = +20 mV, and is such that K^+ is driven to the *trans* compartment. After washout of the *cis* compartment with a proteolipid-free KCl solution, both the membrane conductance (measured at low voltages) and the current fluctuations remained unchanged, indicating that the interaction of the proteolipid with the bilayer is irreversible. The membrane

conductance, 2.1×10^{-7} S/cm^2 (measured at small applied potentials), corresponds to the conductance of a bilayer in symmetrical KCl and exposed to the same concentration of proteolipid as the one used (e.g., Fig. 5). Figure 6*B* shows the current fluctuations when KCl was replaced by NaCl in the *cis* compartment and for a voltage of +20 mV. It is obvious from this record that the amplitude of the current steps is smaller than those seen in the record in 6*A*. Figure 6*C* corresponds to KCl replacement of NaCl in the *cis* compartment and shows that the amplitude of the current fluctuations is indeed a function of the cation which is carrying the current (*c.f.* Fig. 6*A*). Figure 6*D* is a record of the current for (V–PD) = −20 mV. In this case, Na^+ is being driven to the *cis* compartment. The size of the fluctuations corresponds with those in Fig. 6*B*, when Na^+ was moving from the *cis* compartment. Records like the one shown in Fig. 6*D* were observed throughout the various changes indicated.

When experiments like the one described above were performed beginning with NaCl in the *cis* compartment, it was found that the current fluctuations when Na^+ was the charge carrying species were smaller than those observed when K^+, the major cation present in the *trans* compartment, was the charge carrier. It was further found that the conductance of the bilayer was the same as the one obtained with NaCl present in both compartments (data not shown).

Figure 7 depicts the distribution of the amplitudes of the single-channel conductance obtained from records such as those shown in Fig. 6. The figure shows that for symmetrical NaCl solutions, the distribution is narrow, with the peak around 5 pS. With KCl in the *cis* compartment and NaCl in the *trans* compartment, the peak is shifted to a higher value (around 15 pS) and the distribution is wider. This widening of the distribution might be due to the presence of different ions in the *cis* and *trans* compartments, since it was found that with symmetrical KCl solutions the distribution was narrower with the peak around 10–15 pS (not shown).

The results shown in Figs. 6 and 7 strongly indicate that the apoproteolipid incorporates into the membrane both in the presence of Na^+ and in the presence of K^+, inducing channels which are about three times more permeable for K^+ than for Na^+. This permeability ratio was also found when measuring the open-circuit potential, which is present when KCl is in one side and NaCl is in the other side of a proteolipid-containing bilayer (e.g., legend to Fig. 6).

The selectivity between cations and anions was measured by measuring the open-circuit potentials due to a salt concentration gradient across the bilayer.

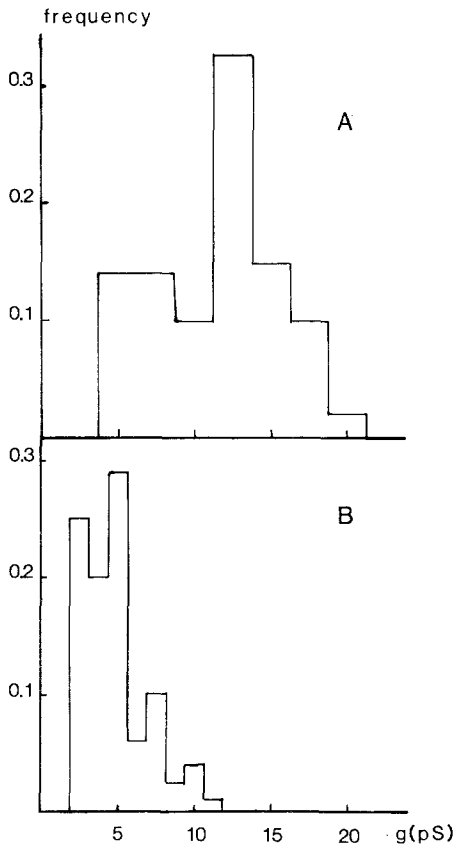

Fig. 7. Distribution of amplitudes of the single-channel conductances. Same membrane as in Fig. 6. Voltage = +20 mV. (*A*): 100 mM KCl in *cis* compartment, 100 mM NaCl in *trans* compartment; number of events counted = 195. (*B*): 100 mM NaCl in *cis* and *trans* compartments; number of events counted = 143

Figure 8 shows a plot of these potentials (PD) as a function of the logarithm of the activity ratio. The sign of the potential indicates that the proteolipid-containing bilayers are more selective for cations than for anions. The slope of the line, 46 mV per 10-fold ratio of salt activity, indicates that K^+ and Na^+ are preferred over Cl^-. The transference numbers for cations (K^+ or Na^+) and Cl^- were found to be: $t_{cat} = 0.90$; $t_{an} = 0.10$ using the following expressions: $PD = \Sigma t_i E_i$ and $\Sigma t_i = 1$, where t_i = transference number of i-th ion and $E_i = (RT/F) \ln a_i^o/a_i^i$ is its equilibrium potential. a_i^o: activity in *trans* compartment; a_i^i: activity in *cis* compartment.

In order to determine if both major fractions of the proteolipid are necessary for the effects described to occur, *FI* and *FII* (Fig. 1) were separated and subsequently tested for their effects on bilayers. Figure 9*A* shows that *FI*, the higher molecular weight component is totally inactive, even at concentrations as high as 12 μg/ml. The lower molecular weight component, *FII*, does interact with bilayers and increases the membrane conductance (e.g., Fig. 10). However, this interaction is not identical to the one observed

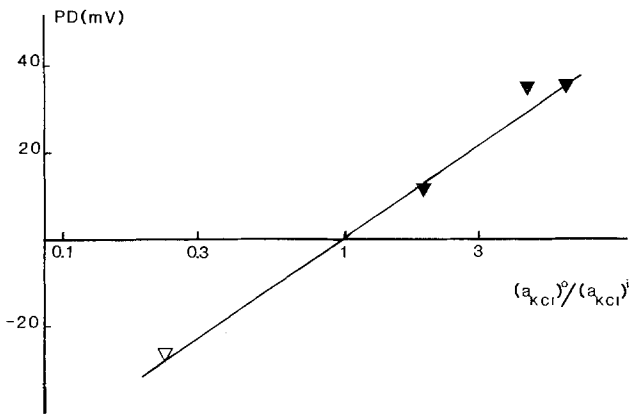

Fig. 8. Trans-membrane potential as a function of the logarithm of the ratio of KCl activities on the two sides of a proteolipid-treated membrane. Bilayers were formed in 20 mM KCl, 5 mM PO_4, pH 7.0, and proteolipid was added to the *cis* compartment (*i*). The KCl concentration was then increased in the *trans* compartment (*o*) and the transmembrane potential determined (▼). ▽: increased KCl concentration in *cis* compartment. Overlapping values were obtained using NaCl instead of KCl

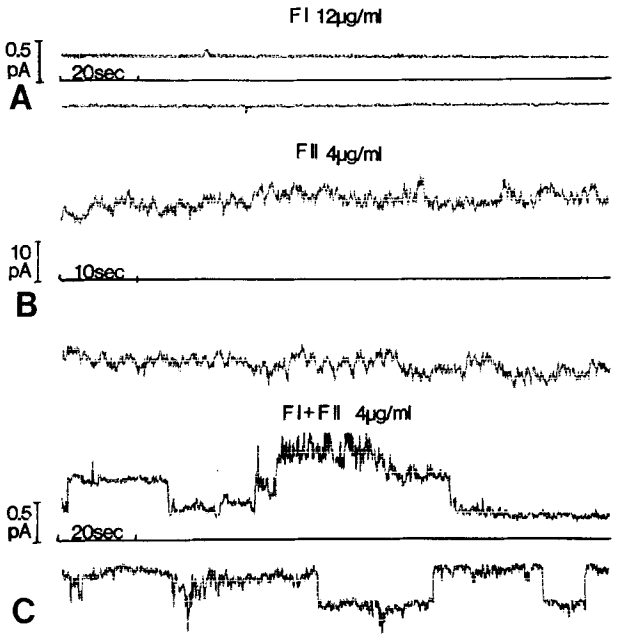

Fig. 9. Time course of membrane current, voltage clamped at + 20 mV. The horizontal lines indicate the zero current level. Membranes were formed in 100 mM KCl, 5 mM PO_4, pH 7.0. (*A*): Fraction *I* was added to *cis* compartment to 12 µg/ml. (*B*): Fraction *II* was added to *cis* compartment to 4 µg/ml. (*C*): Mixed (*FI+FII*) (3:1 weight ratio) were added to *cis* compartment to a total protein concentration of 4 µg/ml

with the unfractionated material since, as illustrated in Fig. 9*B*, no discrete current fluctuations like those shown in Figs. 3 and 4 could be observed. Furthermore, the dependence of the zero voltage conductance on protein concentration (Fig. 10) also differs from the one obtained with the unfractionated material in that the conductance levels obtained at the same protein concentration are lower for *FII* and the conductance shows signs of saturation as the concentration of *FII* is increased.

When *FI* and *FII* were mixed in a 3:1 weight ratio (*FI/FII*), the discrete current fluctuations were seen again (Fig. 9*C*), but the conductance of the bilayer did not increase. Increasing the proportion of *FII* in the mixture to a final 3:1 weight ratio (*FII/FI*), the weight ratio of the fractions in the extract, leads only to a modest increase in the membrane conductance. Thus, these results indicate that the two fractions we have tested either do not contain all of the kinds of molecules responsible for the effects of the whole extract, or that the process of isolation alters the components in the fractions so that they can no longer interact with the bilayer in the same way that they did before the procedure.

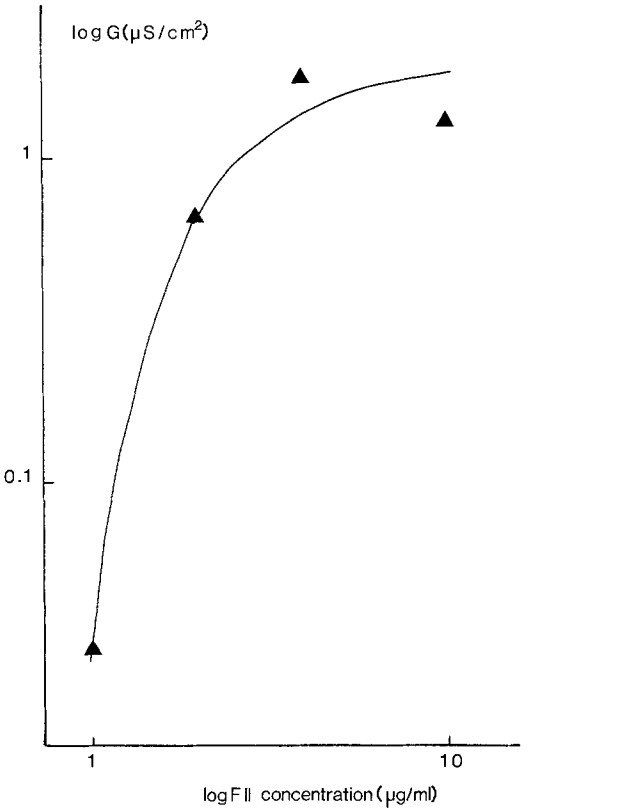

Fig. 10. Double logarithmic plot of membrane conductance *vs.* fraction *II* concentration. Membranes were formed in symmetrical salt solutions: 100 mM KCl, 5 mM PO_4, pH 7.0. Successive additions of *FII* were made to one compartment

Discussion

The data reported in this paper show that the proteolipids isolated from bovine kidney plasma membrane interact irreversibly with bilayers made from diphytanoylphosphatidyl choline. The incorporated proteolipids form channels which are more selective for cations than anions (e.g., Fig. 8) and which are about

three times more permeable to K^+ than to Na^+ (e.g., Figs. 3 and 4). At a fixed protein concentration, the ratio of the membrane conductance in KCl to the conductance in NaCl is $>10^2$ (e.g., Fig. 5). This result probably reflects a difference in the water-membrane partition of the proteolipid in the two different salts which might arise from different, ion-dependent conformations of the protein. Although there are no reports of such an ion-dependent conformational change of the kidney proteolipid, there are reports in the literature on the ion-dependent incorporation of proteins and peptides into bilayers [17, 20]. Of particular relevance to the present study are the results of Ting-Beall et al. [20] on the incorporation of the proteolipid apoprotein from brain white matter. They have reported that the myelin proteolipid induces a voltage-dependent conductance in bilayers only in the presence of Na^+. Once incorporation occurred, however, the modified bilayers did not show selectivity for Na^+ over K^+ as judged by the absence of a transmembrane potential when equimolar amounts of NaCl and KCl were present on opposite sides of the proteolipid-containing bilayer. In contrast to Ting-Beall's result, the same type of measurements show that the proteolipid used in the present study, isolated from bovine kidney, renders the bilayers more selective to K^+ than to Na^+ (e.g., legend to Fig. 6). Moreover, this selectivity was found to correspond to the selectivity of the channels which are induced by the proteolipid both at low (i.e., $\leqq 0.6$ μg/ml) and at higher (i.e., >1 μg/ml) protein concentrations (e.g., Figs. 3 and 4). The reason for the different results obtained with the proteolipids from brain white matter and from kidney plasma membrane probably lies in the differences in the structure of these proteins. Since the amino acid composition of the two preparations is remarkably similar and the main differences being the lack of half cysteine and the absence of bound fatty acids in the kidney apoproteolipids [12], any further speculation concerning the differences in mode of action of the CNS and the kidney proteolipids should be postponed until more is known about the structure of these proteins.

When the starting material which had been used throughout was separated in its two major fractions (e.g., Fig. 2) and tested in bilayers, it was found that neither *FI* nor *FII* induced changes in the membrane like those promoted by the unfractionated material. Some, but not all, of the characteristics were recovered upon aqueous mixing of the two fractions (e.g., Fig. 9). These results could be explained by the lack of some crucial component in the isolated fractions. Alternatively, the inability to reproduce the results obtained with the starting material after mixing of the components could be attributed to a difference in the conformation of the proteins (*FI* and *FII*) once separated. This new conformation might not be as conducive to interaction with the bilayer as the conformation which occurs before separation. Work is currently in progress to determine which of the hypotheses is correct and to try to establish conditions under which successful reconstitution of the original complex can occur.

The nature of the two seemingly different channel populations which are present at different proteolipid concentrations is not clear. At low protein concentrations ($\leqq 0.6$ μg/ml), there seems to be one population of channels with only two states open or closed (e.g., Fig. 3). As the protein concentration is increased, the number of channels increases and so does the steady-state membrane conductance (e.g., Fig. 5). When the concentration of channel-forming molecules in the membrane reaches an apparently "critical" value, new discrete channels appear. These channels have a larger conductance, are voltage-dependent (e.g., Fig. 4), and probably arise as a consequence of an oligomerization of the proteolipid in the membrane. The existence of such oligomers is supported indirectly by data like that illustrated in Fig. 2*A* which shows oligomeric forms of the proteolipid when solubilized in SDS directly from chloroform/methanol. The membrane concentration of proteolipid at which these new channels appear is probably the same whether the aqueous solutions contain NaCl or KCl. The fact that more protein is required in the aqueous phase to see the channels in NaCl (e.g., Fig. 4) is probably a consequence of the lower partition into the bilayer of the channel-forming molecules when in this aqueous medium. The voltage dependence of the channels might be due to a voltage-induced change in the equilibrium distribution between different conformations of aggregates as is the case for alamethicin and suzukacillin [8, 10]. Work is currently in progress to find answers to this and other questions left open by the investigations reported in this paper.

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