Conductance Properties of Artificial Lipidic Membranes Containing a Proteolipid from *Electrophorus*

Response to cholinergic agents

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ABSTRACT Previous studies have shown that a special proteolipid extract from the electric organ of *Electrophorus* showed high affinity binding for acetylcholine and other cholinergic agents. This proteolipid has now been incorporated into ultrathin lipidic membranes, and the membrane resistance was studied. The resistance decreased from $7.27 \pm 0.82 \times 10^5$ ohm cm^2 in the control membrane to 1.83×10^5 ohm cm^2 with addition of 72 μg/ml proteolipid. The decrease in resistance followed a potential function of order four with the proteolipid concentration in the membrane-forming solution. The presence of this proteolipid determined some type of cationic selectivity which was not observed in the control. At a critical point of proteolipid concentration the conductance spontaneously fluctuated between two levels. The membrane current jumped from one state to another by way of single discrete steps, reminiscent of those obtained with the excitatory inducing material or the macrocyclic antibiotics. In membranes containing another proteolipid having no cholinergic binding properties, the increase in conductance was smaller, and had a linear function with the concentration. In this case the "flip flop" fluctuation and the cationic selectivity were not observed. The membranes containing the cholinergic proteolipid reacted to the addition of acetylcholine by a rapid and transient increase in conductance that was considerably reduced or abolished by a previous application of *d*-tubocurarine. These membranes also interacted with other cholinergic agents, such as gallamine triethiodide, hexamethonium, and α-bungarotoxin. These results suggest that this special proteolipid, when added to the artificial membranes, induces a "chemical excitability" toward cholinergic ligands.

INTRODUCTION

The mechanism of chemical synaptic transmission implies the existence of a specific protein receptor at the postsynaptic membrane. It also presupposes that the interaction between the transmitter released by the nerve ending and the receptor results in a change of ionic permeability, which may either depolarize or hyperpolarize the membrane. Previous studies from this laboratory have shown that special proteolipids extracted from the nerve ending membranes of the cerebral cortex and other gray areas of brain had the property of interacting with dimethyl-*d*-tubocurarine (1, 2), serotonin (3), and atropine (4). Furthermore, a special proteolipid extracted from the electric organ of *Torpedo* and *Electrophorus* showed high affinity binding for acetylcholine and other cholinergic agents (5).

The present paper describes experiments in which the cholinergic proteolipid extracted from *Electrophorus* was incorporated into artificial lipidic membranes, similar to those first described by Mueller et al. (6). The results obtained indicate that an artificial membrane containing this proteolipid could have a certain ionic selectivity and that its conductance can be considerably changed by the addition of some cholinergic agents. A preliminary account of some of these findings has been published (7).

MATERIALS AND METHODS

Artificial membranes were formed using a solution of chloroform-methanol-tetradecane (1.0:0.8:0.4) containing 10 mg/ml of synthetic dipalmitoyl-DL-α-lecithin (Sigma Chemical Co., St. Louis, Mo.) or 10 mg/ml of total phospholipids from bovine cerebral cortex extracted according to Folch et al. (8). In this last case the solvent of the lipid extract was evaporated several times to precipitate the proteolipids and then the phospholipids were separated and purified through a column of silicic acid. The resulting material was a mixture of the phospholipids normally present in bovine cerebral cortex. In all cases the membrane-forming solution contained 10 mg/ml of synthetic cholesterol (Sigma Chemical Co., St. Louis, Mo., 99+ %).

The lipid proteolipid membrane was made using the same solution of lipids into which the proteolipid from *Electrophorus electricus* was added to a final concentration of 5–80 μg protein/ml. Two of the five proteolipid peaks that are separated from the total lipid extract of the electric organ by column chromatography in Sephadex LH-20 (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) were used: peak I, that has no binding capacity for acetylcholine, and peak III, the so-called "receptor" proteolipid, which shows the high affinity for binding cholinergic agents (4).

Two different experimental setups were used. In one of them membranes were made across a 1 mm diameter hole, in a vertical Teflon septum separating two chambers containing in most cases a saline with 100 mM NaCl and 50 mM tris(hydroxymethyl)aminomethane (Tris)-Cl buffer (pH 7.0). The bathing solutions were stirred

with a small helix of Teflon electrically driven. The speed of the helix was between 16 and 45 rpm. In some cases the membranes were made in a horizontal Teflon septum (9). This last setup is a modification of the most common type in which the septum is vertical. Besides this, the rest of the system was the same. Membranes made in either vertical or horizontal septa showed similar characteristics.

The ionic composition of the saline was changed in certain cases and this will be mentioned in the Results. The pattern of membrane formation was checked with a stereomicroscope and black membranes were obtained within 5–10 min after applying the forming solution with a brush.

A voltage difference across the membrane was maintained constant by a DC source and it was measured, via calomel electrodes, with a Keithley DC Voltmeter 200 B (Keithley Instruments, Inc., Cleveland, Ohio). The current was determined with a Keithley 150 A Microammeter and recorded with a Heath EUW Servo-Recorder (Heath Co., Benton Harbor, Mich.). Since the applied voltage was maintained constant, the DC change reflected a variation in membrane conductance (10).

Different pharmacological agents were added with capillary tubes (Kimax 34500; size 1.5–2.0 × 100 mm) containing 50–100 μl of solution. In most cases the drugs were applied on the surface of the membrane. The tube formed a 45° angle with the membrane at a distance of 3 mm from it and at the moment of injection the liquid column fell by gravity. In other experiments the drugs were added to the bathing solution as far as possible from the membrane. In this case, under stirring, the concentration of the drug was gradually raised up to a uniform final concentration in the chamber (see Results). In all cases the application of the drugs was made on the positive side of the membrane under an applied voltage of 50 mv.

All experiments were performed at 22°C.

RESULTS

I. *Conductance Changes Resulting from the Addition of the Cholinergic Proteolipid into Lipidic Membranes*

(A) MODIFICATION OF THE RESTING CONDUCTANCE In control membranes (i.e., without proteolipid), the current-voltage (I/V) curves showed an ohmic relation between 0 and ±100 mv and their resting resistance was 7.27 ± 0.82 × 10^5 ohm cm^2 with synthetic phosphatidylcholine as the amphipatic molecule. This value is similar to those reported in the literature (11).

The two proteolipids from *Electrophorus* (i.e., peaks I and III), when incorporated into the membrane-forming solution, increased the membrane conductance (Fig. 1). In membranes containing synthetic phosphatidylcholine, the presence of 58 μg/ml of proteolipid from peak I reduced the resistance from 7.27 ± 0.82 × 10^5 to 3.54 ± 0.42 × 10^5 ohm cm^2 (n = 8). Similarly, the presence of 72 μg/ml of the cholinergic proteolipid (peak III) lowered the resistance, measured at 100 mv, to 1.83 ± 0.15 × 10^5 ohm cm^2 (n = 8). In this last case the I/V plot deviated slightly from linearity above 75 mv. This was an indication of the beginning of a rectification which increased even more with voltages greater than 100 mv.

An important difference in conductance was observed by the addition of increasing concentrations of these two proteolipids. In the case of the cholinergic proteolipid a potential relationship of order four between the in-

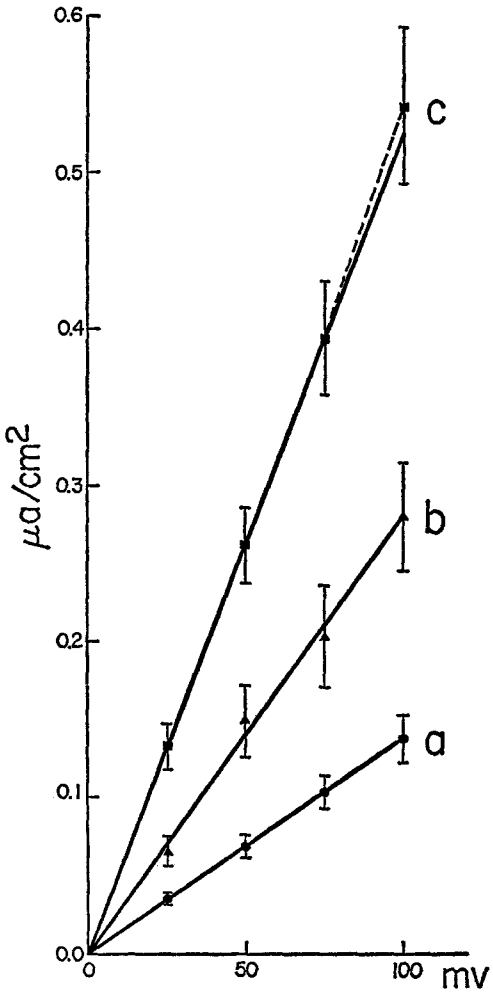

FIGURE 1. Membrane current as a function of the applied voltage. (*a*) control (containing synthetic phosphatidylcholine as the amphipatic molecule); (*b*) control plus proteolipid of peak I; (*c*) control plus proteolipid of peak III. The membranes were formed in 100 mM NaCl and 50 mM Tris-Cl buffer.

crease in conductance and the concentration of proteolipid was established. Fig. 2 shows a log-log plot of these two parameters with a slope of four. This finding suggests the possibility that the conductance change induced by the cholinergic proteolipid follows a cooperative type of function. With the noncholinergic peak I such a relationship was not obtained, and the con-

ductance increased in a more or less linear manner with the proteolipid concentration.

(B) DEPENDENCE OF THE RESTING CONDUCTANCE ON THE CATIONS PRESENT IN THE BATHING SOLUTIONS Although the mechanism by which ions may be translocated across artificial lipidic membranes is not well understood several investigators have found that they lack cationic selectivity; in other words

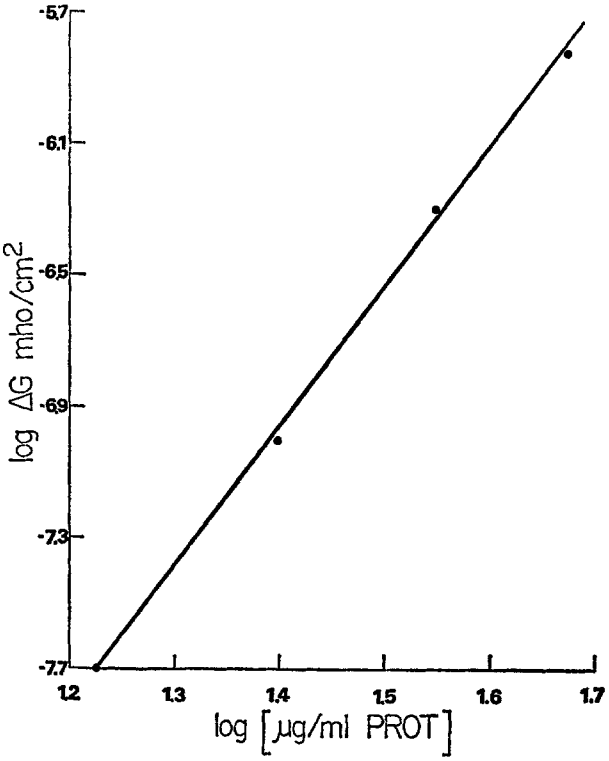

FIGURE 2. Relationship between the log of the conductance change of the membrane and the log of the proteolipid concentration in the membrane-forming solution. Total phospholipids from bovine cerebral cortex were used in this experiment.

the numbers of cationic transference are similar for different cations (11). It is also known that some macrocyclic antibiotics such as valinomycin, when added to the lipidic membranes, induce in them a cationic selectivity. It was thus of interest to determine if the incorporation of the proteolipid could develop some type of specific permeability in the artificial membrane.

A first approach to this problem was to observe the variation in the I/V curve when NaCl was removed and replaced by CsCl. The I/V curves of Fig. 3 A show that the removal of the NaCl in control membranes resulted in an increase in resistance from $7.27 \pm 0.82 \times 10^6$ to $1.16 \pm 0.91 \times 10^6$

ohm cm² at 100 mv, which could be compensated almost to the initial value (7.32 ± 0.70 × 10^5 ohm cm²) with the addition of CsCl. The I/V curves of Fig. 3 B demonstrate that the introduction of the cholinergic proteolipid results in a considerable decrease in resistance (2.5 ± 0.18 × 10^5 ohm cm²) which is raised (3.65 ± 0.20 × 10^5 ohm cm²) by the removal of the NaCl. It may also be observed that the replacement with CsCl does not result in a restoration of the initial resistance, which is an indication that the cholinergic

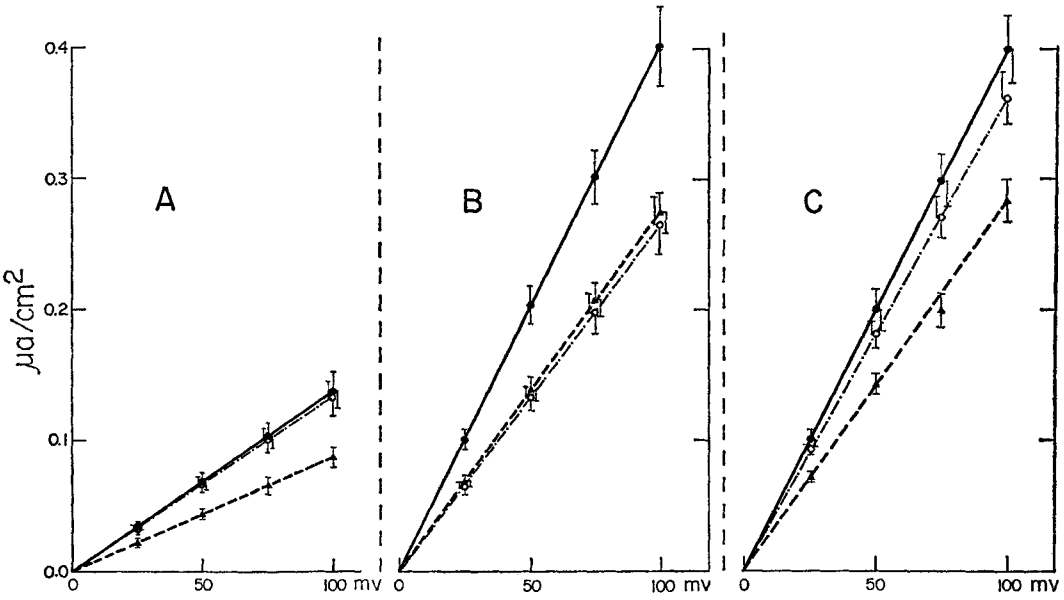

FIGURE 3. Membrane current as a function of the applied voltage. (A) control membrane (with synthetic phosphatidylcholine). (B) control membrane plus proteolipid from peak III. (C) control membrane plus proteolipid from peak I. -●—●—●-, in the presence of 100 mM NaCl and 50 mM Tris-Cl buffer. -o—·—o—·—o-, in the presence of 100 mM CsCl and 50 mM Tris-Cl buffer. -▲—▲—▲-, in the presence of 50 mM Tris-Cl buffer alone.

proteolipid has induced some sort of cationic selectivity. On the other hand the I/V curves of membranes containing the nonreceptor proteolipid (peak I) did not show differences between sodium and cesium (Fig. 3 C). Similar experiments were performed by replacing NaCl by KCl, LiCl, or RbCl. Table I shows the ratio between the current flowing through the membrane in the presence of different cations of group I A and in the presence of Na. These values agree with one of the series (No. VII) of cationic selectivity proposed by Eisenman (12).

(c) THE DEVELOPMENT OF A BISTABLE CONDUCTANCE STATE Another effect of the addition of increasing concentrations of cholinergic proteolipid

to the membrane-forming solution was that of introducing two alternative conductance levels (9). We again observed that in certain membranes the conductance became bistable, switching reversibly in discrete steps from a low to a high conductance state (Fig. 4 A). Similar fluctuations have been previously observed when the excitability inducing material (EIM) (10, 13) or alamethicin (14) was added to artificial lipidic membranes. These fluctuations have been interpreted as being caused by the opening and closing of conducting channels (10).

TABLE I

RATIO BETWEEN THE CURRENT FLOWING THROUGH THE MEMBRANE IN THE PRESENCE OF NaCl AND IN THE PRESENCE OF THE CHLORIDE SALTS OF DIFFERENT CATIONS FROM GROUP IA

Compared cations	Current ratio
LiCl/NaCl	0.76±0.09
KCl/NaCl	1.09±0.11
RbCl/NaCl	0.98±0.10
CsCl/NaCl	0.66±0.07

Each pair of experiments comparing every cation to sodium was done with the same membrane-forming solution, which in all cases contained synthetic phosphatidylcholine as the amphipatic molecule. Mean of 10 experiments in each case.

II. *Conductance Changes Resulting from the Injection of Cholinergic Agents* As described above different pharmacological agents were added to the positive side of the artificial membrane.

(A) THE RESPONSE TO ACETYLCHOLINE The injection of acetylcholine chloride (Sigma Chemical Co., vials), upon membranes containing proteolipid from peak III and synthetic phosphatidylcholine produced a rapid and transient increase in DC current intensity (Figs. 4 C and 5). Since the applied voltage is maintained constant, the DC effect reflects an increase in the conductance of the membrane. When acetylcholine was added to control membranes made with synthetic phosphatidylcholine (Fig. 4 B), or to those containing the "nonreceptor" proteolipid (peak I) plus phosphatidylcholine, in no case was an increase in conductance detected. A similar response to acetylcholine was obtained when phospholipids from the cerebral cortex were mixed with proteolipids from peak III.

An important question was whether the response is transient by itself or because the material diffuses away from the membrane. The speed of mixing played a role in the kinetics of the response. In Fig. 4 C we can see the effect of stirring the bath solution. The magnitude of the response with or without

agitation was the same, but the rate of return to base line was faster, improving the mixing conditions. However the transient characteristic of the response is not completely due to a diffusion mechanism. When the acetyl-

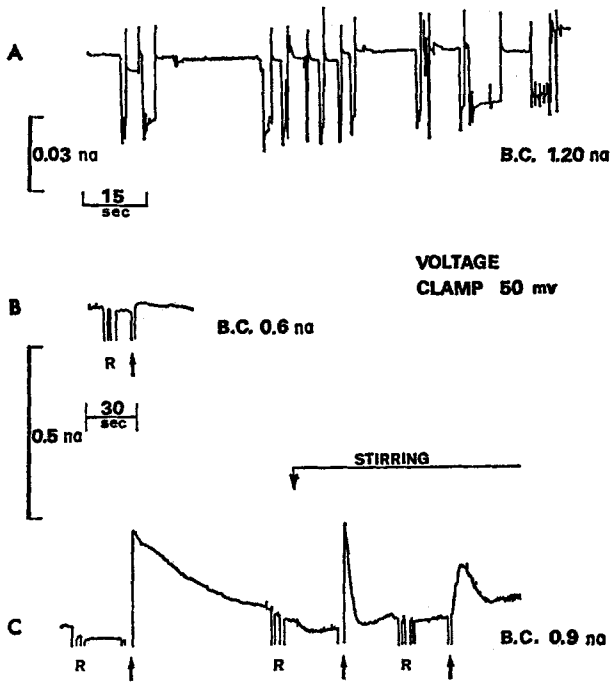

FIGURE 4. Original records of membrane current as a function of time. The experiments were performed in 100 mM NaCl and 50 mM Tris-Cl buffer. Voltage clamp: 50 mv; *B.C.*: base-line current. (A) Spontaneous current fluctuations observed with a critical concentration of cholinergic proteolipid. In this case the amphipatic molecule was synthetic phosphatidylcholine. (B) Effect on membrane current induced by the application of acetylcholine chloride (arrow) on a control membrane without proteolipid. (C) Transient changes in membrane current induced by the application of acetylcholine chloride (arrows) on a membrane with proteolipid from peak III. First arrow: response to 100 μl of 5×10^{-2} M acetylcholine injected on the surface of the membrane without stirring. Second arrow: identical injection but the bath solution was stirred (45 rpm). Third arrow: the acetylcholine concentration in the bath was raised to a uniform concentration of 5×10^{-2} M (stirring; 45 rpm). Previous to each injection of acetylcholine (arrows) a volume similar to that to be injected was removed (*R*) in order to preserve the volume constant.

choline concentration increased gradually up to 5×10^{-2} M a transient variation in conductance was also observed (Fig. 4 C) but the steady-state value of the conductance increased in this experiment. In this last type of experiment, if the rate of increase in acetylcholine concentration is reduced, the magnitude of the transient response diminishes, even if the final concen-

tration of acetylcholine is the same. It seems that the magnitude of the response is related to the rate of change in acetylcholine concentration in the vicinity of the membrane.

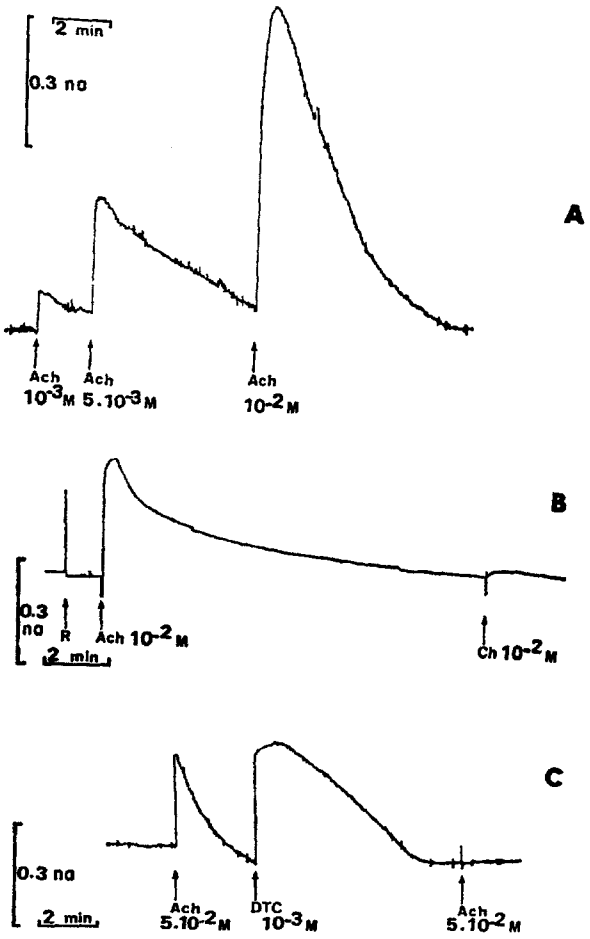

FIGURE 5. Original records showing the response of membranes containing the proteolipid from peak III to cholinergic agents (*Ach*). (A) Responses to different doses of acetylcholine chloride. *B.C.*: 0.82 na. (B) Responses to saline (*R*), acetylcholine chloride, and choline chloride. The membrane-forming solution contained synthetic phosphatidylcholine. *B.C.*: 1.01 na. (C) Interaction between acetylcholine chloride and dimethyl-*d*-tubocurarine chloride (*DTC*). *B.C.*: 0.85 na.

The membrane with total phospholipids from bovine cerebral cortex had a lower control resistance than those with synthetic phosphatidylcholine and the variation in conductance induced by acetylcholine was greater, the membrane current increasing up to five times. However, there were great variations between different cases, and in control membranes (with no

proteolipid from *Electrophorus*) a small response was obtained in some cases, with large doses of acetylcholine. This may be due to the presence in the total phospholipid extract from cerebral cortex of some contaminating cholinergic proteolipid. (The presence of cholinergic proteolipids in the cerebral cortex has been previously described [1, 4].) Therefore we decided to use, in most pharmacological experiments, synthetic phosphatidylcholine. With this phospholipid as the amphipatic molecule the reproducibility of the response to acetylcholine in the presence of proteolipid from peak III was excellent. Th magnitude of the increase in membrane current was up to 100% of the base-line current.

The response obtained with the cholinergic peak was dependent on the acetylcholine concentration in the pipette (Fig. 5 A). However, there was a variation in sensitivity between cholinergic proteolipids obtained in different extractions and in a few cases no response could be obtained. Since we know from previous experience that in the column of Sephadex LH-20 peak III always binds acetylcholine we have no explanation for the failure with some extracts. Employing in all the experiments the same membrane-forming solution made with a cholinergic proteolipid that showed a good reactivity, a dose-response curve was obtained with different concentrations of acetylcholine (Fig. 6). We also correlated the amount of cholinergic proteolipid present in the membrane with the response to a single concentration of acetylcholine (in this case 5×10^{-2} M). The response increased with the concentration of the proteolipid in the membrane-forming solution but it was not possible to establish the exact type of function relating these two parameters (Fig. 7).

Control experiments were carried out with choline chloride applied in similar concentrations either to lipidic membranes or to those containing proteolipids from peak I and peak III. In no case was there a transient response similar to that elicited by acetylcholine (Fig. 5 B). Also, no responses were obtained with sodium acetate in concentrations as high as 10^{-1} M. To eliminate the influence of changes in the ionic concentration, control experiments were performed injecting different solutions. With distilled water, 300 mM NaCl, or 300 mM KCl, no significant changes in conductance were observed.

(B) THE ACTION OF *d*-TUBOCURARINE; INTERACTION WITH ACETYLCHOLINE

The injection of *d*-tubocurarine (10^{-3} M) also elicited a transient increase in conductance (Fig. 5 C). However this response did not show the same specificity as acetylcholine. In fact control membranes made with brain phospholipids or with synthetic lecithin and membranes containing proteolipid peak I also reacted with *d*-tubocurarine. However an important difference in connection with the action of acetylcholine was observed. The transient conductance increase induced by acetylcholine in membranes con-

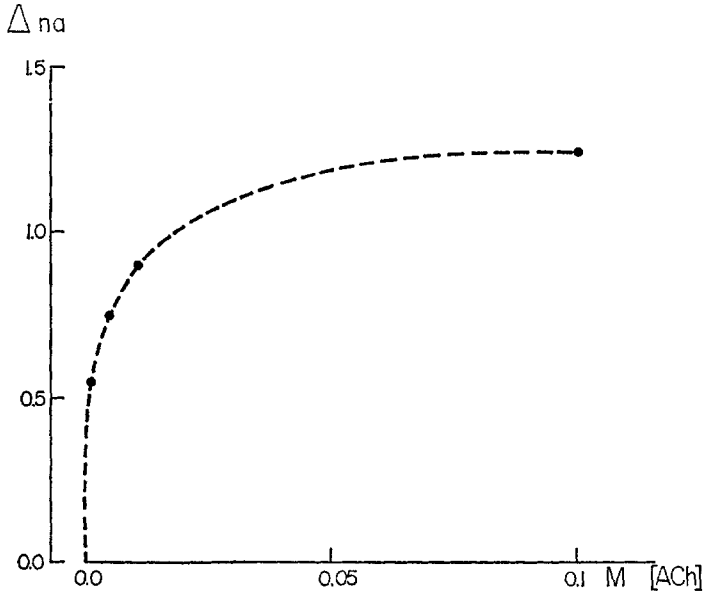

FIGURE 6. Dose-response curve obtained in a single membrane using increasing molar concentrations of acetylcholine chloride in the pipette. The abscissa represents the current increase induced by the drug at the peak of the response. The experiment was performed in 100 mM NaCl and 50 mM Tris-Cl buffer.

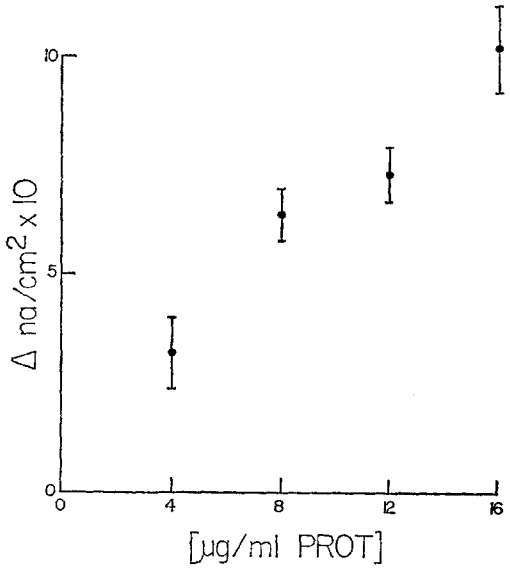

FIGURE 7. Relationship between the increase in membrane current induced by an injection of a 5×10^{-2} M solution of acetylcholine chloride and the proteolipid concentration in the membrane-forming solution containing synthetic phosphatidylcholine. The current increase was measured at the peak of the response. All experiments were performed in 100 mM NaCl and 50 mM Tris-Cl buffer.

taining the cholinergic proteolipid (peak III) could be repeated by successive injections on the same membrane, but after *d*-tubocurarine the reactions to acetylcholine were much reduced, or disappeared (Fig. 5 C).

It has been previously described that with membranes containing the "nonreceptor" peak I and brain phospholipids a small "response" could be obtained at high concentrations of acetylcholine. In this case the application of *d*-tubocurarine did not reduce the response.

(C) ACTION OF OTHER CHOLINERGIC AGENTS The injection of Flaxedil (Rhône Poulenc, Rhodia, Buenos Aires) (gallamine triethiodide) upon artificial membranes gave a response somewhat different than that produced by *d*-tubocurarine. As shown in Fig. 8 A1 the application of 10^{-2} M Flaxedil in the pipette produced a transient decrease in membrane conductance which was not specific for the cholinergic proteolipid since it was also seen in control membranes and in those containing peak I. In spite of these findings an interaction between Flaxedil and acetylcholine for the cholinergic proteolipid could be demonstrated. As shown in Fig. 8 A2 while the reaction of this proteolipid to acetylcholine is rapid and the maximal deflection is obtained in 2–3 sec, with the previous application of Flaxedil the conductance increased slowly and the maximum change was observed in 60 sec. Furthermore, when Flaxedil was added at the peak of the acetylcholine reaction there was an immediate reversal of the response (Fig. 8 A3). When 100 μl of the saline solution was injected at the peak of the acetylcholine reaction only a small and transient reduction in conductance was observed, but in no case did it fall to the basal level.

Hexamethonium had no effect upon the membrane conductance but when added after the application of acetylcholine it immediately reduced the conductance to the control level (Fig. 8 B).

α-bungarotoxin, a polypeptide of 8000 mol weight extracted from the venom of the snake *Bungarus multicintus*, produces a permanent blockade of cholinergic synaptic transmission (15). As shown in Fig. 8 C1 this toxin produces no effect on artificial membranes containing phosphatidylcholine. On the other hand, when α-bungarotoxin was applied to the membrane containing the cholinergic proteolipid there was a slow increase in conductance until the membrane broke. In Fig. 8 C2 it may be observed that this effect was dose-dependent and became evident with concentrations above 10^{-4} M in the pipette. The finding that the injection of 10^{-4} M α-bungarotoxin did not change the conductance permitted the study of its effect on the response to acetylcholine. In Fig. 8 C3 it may be observed that in a way similar to that of Flaxedil the kinetics of the response were changed and instead of a maximal conductance rise in 2-3 sec it took about 80 sec for the response to be fully developed.

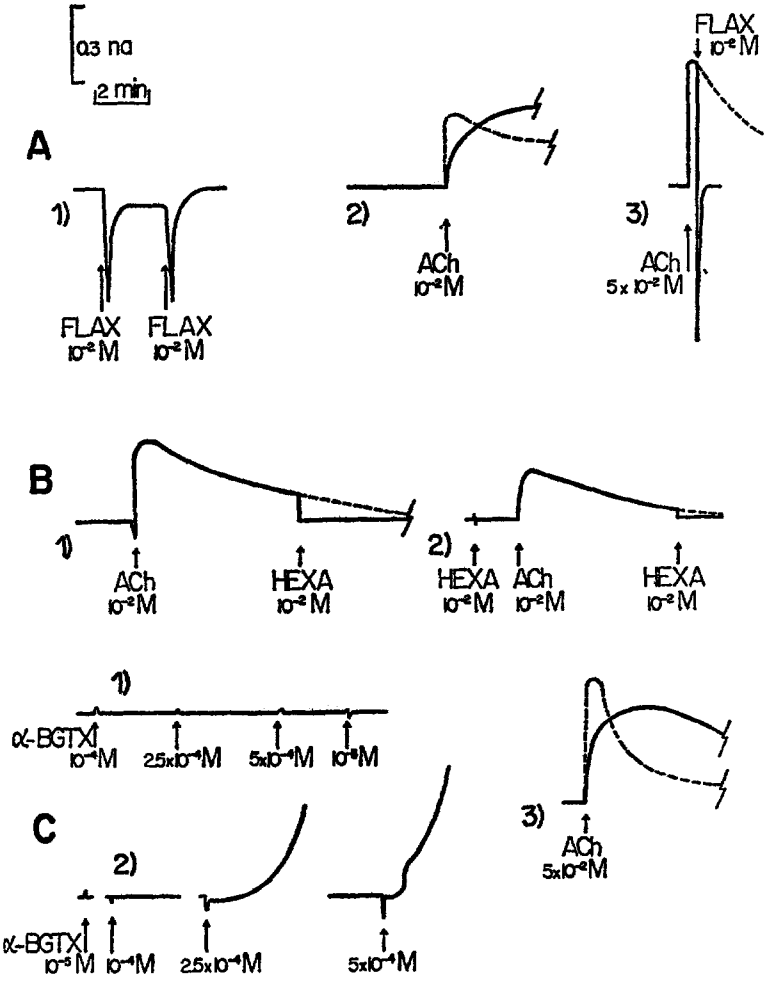

FIGURE 8. Interactions between acetylcholine chloride (*ACh*) and other cholinergic agents. (A) application of Flaxedil (gallamine triethiodide) (*FLAX*): *1*) upon a control membrane without proteolipid; *2*) response to acetylcholine after the injection of Flaxedil; *3*) addition of Flaxedil at the peak of the acetylcholine reaction. In Fig. 8 A *2* and *3* the dotted line represents the normal response to acetylcholine. (B) *1*) addition of hexamethonium (*HEXA*) after the application of acetylcholine chloride; *2*) addition of hexamethonium before and after the application of acetylcholine chloride. The dotted lines represent the normal response to acetylcholine. (C) *1*) application of α-bungarotoxin (*α-BGTX*) in a control membrane without proteolipid; *2*) increase in conductance and membrane breakdown in membranes with proteolipid from peak III; *3*) response to acetylcholine after the injection of α-bungarotoxin. The dotted line in C*3* indicates the normal response to acetylcholine. All experiments were performed under a voltage clamp of 50 mv.

DISCUSSION

The results described here may be discussed with two different approaches. One is to consider the changes that occur in lipidic artificial membranes when some proteolipids extracted from the electric organ of *Electrophorus electricus* are incorporated. The second is to elaborate on the changes that take place when the cholinergic proteolipid of *Electrophorus* (5) present in the lipidic membrane interacts with the cholinergic agents.

It is here demonstrated that the introduction of small amounts of a proteolipid into the membrane-forming solution results in an artificial "black" membrane with a reduced resistance as compared to the control containing only phospholipids and cholesterol.

Previous work from this laboratory (9) has made possible the study of the surface fine structure of these artificial membranes. Using glutaraldehyde fixation followed by osmium tetroxide vapors, it was found that the artificial membranes made only of phospholipids from the cerebral cortex and cholesterol (control membranes) frequently had a globular fine structure with lipid micelles of about 40 A diameter. It was also found that the introduction of small amounts of proteolipids produced a lower electron opacity and a smoother texture of the membrane. These findings suggested that the lower resistance had resulted from a molecular reorganization of the membrane due to the presence of the hydrophobic protein.

In the case of the cholinergic proteolipid the reduction of resistance was accompanied by other interesting biophysical phenomena such as the appearance of some type of "cationic selectivity" favorable to sodium and potassium and the development of bistable conductance states which were not found either in control membranes or in those containing a noncholinergic proteolipid. These findings are reminiscent of those previously observed with a proteinaceous material released by *Aerobacter cloacae* ATCC 961, called by Mueller et al. (13) the excitability inducing material (EIM), or with different polypeptide macrocyclic antibiotics (14). These substances, when added to the artificial lipidic membrane, induce an increase in conductance which is accompanied by ionic selectivity and instability with discrete current jumps, which have been interpreted as being due to the opening and closing of conducting channels (10). Here, for the first time, a protein extracted from the original cholinergic tissue and present in the electroplax membrane (16) is found to have a behavior in artificial membranes somewhat similar to the EIM or the macrocyclic ionophores whose biological roles are unknown. In a recent work Papahadjopoulus (17) has reported an Na^+-K^+ discrimination in "pure" phospholipid vesicles containing phosphatidylserine as the amphipatic molecule. With other phospholipids, including

phosphatidylcholine, the lack of discrimination previously observed (11) was confirmed.

Of special interest seems to be the fact that the increase in proteolipid concentration in the membrane-forming solution not only increases the chances of producing bistable states but also that it bears with the conductance a potential relationship of order four (Fig. 2). De Robertis (18) has proposed a model for the cholinergic receptor in the postsynaptic membrane in which each receptor area is thought to be the result of the tetrameric arrangement of four proteolipid molecules (see Fig. 6 in reference 18). Each of these molecules interacts with a single acetylcholine molecule at the receptor site situated on the surface of the membrane. The rest of the four rod-shaped proteolipid molecules, disposed in parallel and traversing the thickness of the lipid bilayer, represents the ionophoric or conducting portion used for the ionic translocation. This type of model gives a logical explanation for the results presented here, but other possible interpretations cannot be excluded. The conducting portion of the system may not necessarily be a pore or hole across the membrane through which water flows hydrodynamically carrying the different ions. On the contrary, the selectivity observed suggests that the channel represents a zone of reduced resistance to specific cations whose mechanism of translocation needs to be further investigated.

In a previous paper Parisi et al. (7) gave the first description of the change in conductance that is produced by the injection of acetylcholine on the positive side of the membrane. It was also shown that choline chloride had no effect and that a noncholinergic proteolipid from the electroplax gave no reaction to acetylcholine. Using the electron microscope Vasquez et al. (9) could show that the membrane "activated" by acetylcholine had a striking change in surface fine structure which accompanied the increase in conductance. The main change consisted in the appearance of dense spots with a maximal diameter of 20 A into which osmium tetroxide was incorporated. This suggested that the translocation of ions was accompanied by the penetration of osmium tetroxide at certain points of the membrane. Most interesting was the finding that all these changes reverted to the control condition with the return of the conductance to the basal level.

In the present investigation the action of acetylcholine was further explored and a dose-response curve with different concentrations of acetylcholine was obtained. Furthermore the conductance change to a single dose of acetylcholine was found to increase with the concentration of proteolipid in the membrane. Of particular interest is the comparison between curare, a typical blocking agent of the electroplax, and other cholinergic agents that may be active in this type of chemical transmission. A blockade of the response to acetylcholine by the previous application of *d*-tubocurarine was demonstrated. However, *d*-tubocurarine produced an unspecific conductance

change on lipidic membranes as well as in membranes containing a non-cholinergic or the cholinergic proteolipid.

The interaction between Flaxedil and acetylcholine for the cholinergic receptor could also be demonstrated. However this drug produced a decrease in membrane conductance in control membranes. Similarly it could be shown that hexamethonium and acetylcholine interact with the cholinergic proteolipid. An interesting finding is that provided by α-bungarotoxin. The fact that at certain concentrations it breaks the membrane containing the cholinergic proteolipid, but not the control membrane, is already an evidence that it reacts with proteolipid receptors. The other evidence refers to the different kinetics of the conductance change by acetylcholine in membranes previously treated with α-bungarotoxin. The binding of this toxin to the cholinergic proteolipid using other methods for the study of proteolipid-ligand interaction has been demonstrated in this laboratory and the data will be published elsewhere.

It may be concluded that the incorporation of minute amounts of a cholinergic proteolipid from electroplax to artificial lipidic membranes results in a decrease of resistance, in the appearance of some type of "cationic selectivity," and sometimes in a bistable state of the membrane which is reminiscent of the effect of EIM or of some ionophoric macrocyclic antibiotics on artificial lipidic membranes. Furthermore the presence of the cholinergic proteolipid confers to the membrane a special "chemical excitability" by which it reacts with a conductance change to the application of acetylcholine. The latter effect could result from the binding of the drug to the high affinity binding site of the proteolipid which could be coordinated with the translocation of ions through the ionophoric or conductive region of the proteolipid. Some of the results obtained may support the hypothesis that each conductive region in the membrane may be formed by the tetrameric arrangement of proteolipid molecules.

The results described in this paper represent a first approach to the study of the properties of artificial membranes containing special proteolipids. Experiments are now under way to determine more precisely the degree of ionic selectivity of these membranes as well as to explore in greater detail and with the use of other pharmacological agents the specificity of the cholinergic response.

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