Fine Structure of Ultrathin Artificial Membranes

I. Changes by Acetylcholine Addition in Lipid Proteolipid Membranes

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Summary. A special technique for the electron-microscope study of the fine structure of ultrathin artificial membranes is described. Membranes made of total phospholipids of the cerebral cortex and cholesterol showed globular elements of 40 Å embedded in a denser and diffuse matrix. These same elements were also seen organized in a periodic banded pattern. Identical patterns were observed with and without supporting films. Lipidic membranes containing small amounts of proteolipid from *Electrophorus* showed a lower electron density, a finer and smoother texture and a decrease in electrical resistance.

Lipidic membranes containing the cholinergic receptor proteolipid from *Electrophorus*, upon addition of acetylcholine, showed a rapid and transient rise in conductance which was accompanied by changes in fine structure, consisting in a more uneven corrugated appearance of the membrane and the presence of dense spots of 20 Å. These results are discussed in relation to the channel hypothesis of ion permeability. It is postulated that the binding of acetylcholine by the receptor proteolipid results in conformational changes in this protein that facilitate the translocation of ions through the membrane.

Thin lipid membranes, separating two aqueous phases were first obtained by Mueller, Rudin, Ti Tien and Westcott (1962 *a, b*; 1963). They assumed that these membranes were made of a simple bimolecular lipid leaflet and this concept was supported by electron-microscope observations of normal sections which showed a 60 to 90 Å thick dense line, with regions suggesting a bilayered structure. Mueller and Rudin (1963, 1968) found that some ionophoric antibiotics and the so-called Excitability Inducing Material (EIM) caused electrical phenomena resembling those observed in excitable membranes. More recently, Parisi, Rivas and De Robertis (1971) studied some electrical properties of artificial lipidic membranes containing a small

amount of a special proteolipid extracted and purified from electric organ of *Electrophorus electricus* (La Torre, Lunt & De Robertis, 1970). After addition of acetylcholine they found a rapid and transient increase in membrane conductance which could be blocked by dimethyl-*d*-tubocurarine.

The present paper describes experiments designed to observe the planar surface of artificial ultrathin membranes made of lipids, mixtures of lipids and proteolipids or of proteolipid alone. It was possible to observe differences in the fine structure of these different membranes and to detect changes in membranes "activated" with acetylcholine.

Materials and Methods

Composition of Membranes

A solution of chloroform-methanol-tetradecane (1.0:0.8:0.4) containing 10 mg/ml of synthetic cholesterol (Sigma 99+%) and 10 mg/ml of total phospholipids from bovine cerebral cortex was used. This phospholipid extract was made according to Folch, Lees and Sloane-Stanley (1957). The solvent of the lipid solutions was evaporated several times to precipitate the proteolipids. 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.

Lipid-proteolipid membranes were made using the same solution of lipids with the addition of proteolipids from *E. electricus* in a proportion of protein to phospholipids of 1:10,000. Two of the five proteolipid peaks that are separated from the total lipid extract of the electric organ by column chromatography on Sephadex LH 20 were used: peak 1, that has no binding capacity for acetylcholine, and peak 3, the so-called "receptor" proteolipid, which shows high affinity for binding cholinergic agents (La Torre *et al.*, 1970). Other membranes were made with proteolipids alone without the addition of lipids.

Membrane Formation and Biophysical Study

The membranes were made across a 1-mm hole in a horizontal teflon septum separating two chambers containing 100 mM NaCl and 50 mM Tris buffer (pH 7) (Fig. 1). This is a modification of the most common set up in which the septum is vertical. However, membranes made in both types of chambers showed similar characteristics. For technical reasons horizontal ones were preferred.

The instrumental apparatus was similar to that of Ehrenstein, Lecar and Nossal (1970). A voltage difference across the membrane was maintained constant by a d-c source, and it was measured, via calomel electrodes, with a Keithley d-c voltmeter 200 B. The current was determined with a Keithley 150 Å microammeter and recorded with a Heat EUW servo-recorder. Since the applied voltage was maintained constant, the d-c change reflected a variation in membrane conductance. Acetylcholine (10^{-2} M) was added in 50 µliters aliquots by means of a fine polyethelene tube ending at about 2 mm of the positively charged side of the membrane. The pattern of membrane formation was checked with a stereomicroscope. Secondary "black" membranes were obtained within 5 to 10 min. For fixation, 2.5 ml of 8% glutaraldehyde were introduced with a syringe in the upper aqueous chamber (positive side), and simultaneously, 2.5 ml of the buffer were extracted with another syringe to maintain the same hydrostatic pressure,
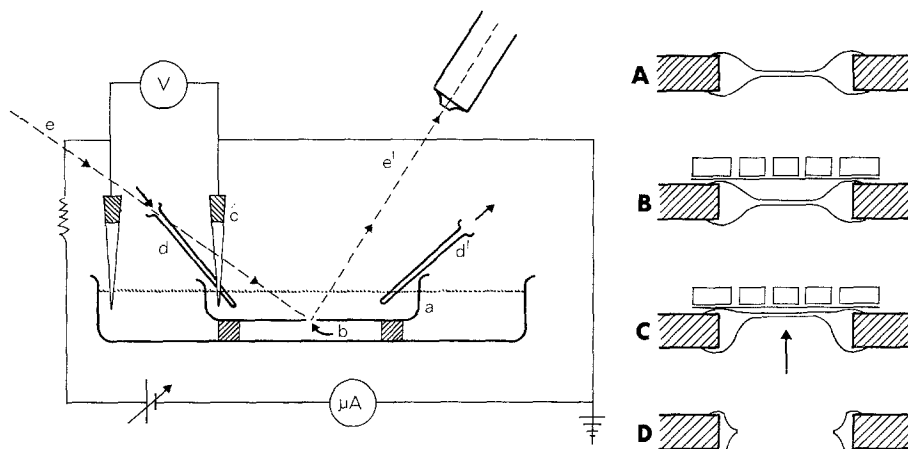
Fig. 1. *Left:* Diagram of the apparatus used to study the electrical properties of artificial membranes. A teflon cup (a) is inmersed in a Petri dish. The membrane is formed in a 1-mm hole at the bottom (b). The electrical measurements are made via calomel electrodes (c). The solution of the upper chamber is changed with the aide of two syringes (d, d'). A light beam, (e, e') reflects on the membrane and is observed with a stereomicroscope. *Right:* The hole in the teflon septum is represented at a larger scale and the different steps to remove the membrane are shown. A, Artificial membrane; B, Grid placed on the hole; C, A hydrostatic pressure sticks the membrane to the grid; D, After removal of the grid some material from the "torus" remains at the edge

thus avoiding breakage of the membrane (Fig. 1). The final glutaraldehyde concentration in the upper aqueous phase was of the order of 2%. All these steps and those following were monitored with the recording instrument.

Electron-Microscopy

After 3 min of glutaraldehyde fixation, a thin carbon film, reinforced with collodion and mounted on a grid (Platinum specimen mounts, Siemens type) was introduced in the upper chamber and carefully deposited on the top of the 1-mm hole with the carbon face of the grid facing the positively charged surface of the membrane (Fig. 1b). Also, empty grids, without supporting films, were used to remove the artificial membranes. Then the upper aqueous phase was slowly evacuated; the change in hydrostatic pressure, bowed the membrane and stuck it to the grid surface (Fig. 1c). During this period the current was recorded to control the membrane integrity. When the upper phase was evacuated, the grid was removed and at this moment the sudden increase in current indicated that the membrane had been removed from the hole (Fig. 1d).

The remaining water on the grid surface was botted with filter paper, and the grid held for 30 sec on osmium tetroxide vapors (1.5% OsO_4). In the carbon-collodion coated grids, the collodion was removed in an oven at 180 °C for 7 min.

A Siemens Elmiskop 1 A electron-microscope was used with an accelerative voltage of 80 kv and a 50-μ objective aperture. The magnifications were calibrated with a standard grating. Astigmatism was fully corrected prior to the use of the microscope and checked every 3 hr. Micrographs were taken at 40,000 magnifications on Kodak Projector Slide Plates.

Results

The mechanical stability of the membrane was increased by the fixation procedure used. Without fixation the membrane transfer to the grid was impossible to achieve; on the other hand, after fixation with glutaraldehyde the membranes were manipulated without difficulty. It is interesting to mention that glutaraldehyde fixation (*see* Fig. 4) only produced a slight change in the level of the normal membrane conductance.

Usually the visible surface of the grid was entirely covered by the artificial membrane. This was particularly well observed when empty grids were used to hold the artificial membranes (Fig. 2).

Lipidic Membranes

Lipidic membranes had a certain degree of electron density after the osmium treatment. The electron micrographs showed large fields in which periodic patterns of dense (30 Å), and clear (40 Å) bands were observed (Fig. 3a). These banded areas were interrupted by others in which globular elements of about 40 Å in diameter predominated (Fig. 3b). In some cases there was the suggestion that the clear bands were formed by the juxtaposition of the globular elements. The same pattern was found either when carbon films or empty grids were used as supports (*c.f.* Fig. 2 with Fig. 3a and b). In these membranes the current voltage (I/V) showed an ohmic relationship between 0 ± 100 mv with a resistance of $4.2 \pm 0.6 \times 10^5$ Ωcm (mean $\pm$ SE, n = 10) (Fig. 4b) and upon the injection of acetylcholine no conductance change was detected.

Lipid-Proteolipidic Membranes

Membranes containing lipids plus proteolipid of peak 1 or 3 spread immediately, making very thin membranes, whose stability was increased upon the glutaraldehyde fixation. After the osmium tetroxide vapors the contrast was quite low. The structure of these membranes was smooth without the bands or globular units observed in the lipidic membranes. The same features were observed using proteolipid from peaks 1 or 3 (Fig. 5). The electrical resistance of these membranes was non-linear between 0 ± 100 mv with values about ten times smaller than that of the lipidic membranes. At 100 mv the mean values were $5.0 \pm 0.9 \times 10^4$ Ωcm^2 (n = 12) (Fig. 4d). In some cases the presence of proteolipid of peak 3 in the membrane induced discrete current jumps which were not observed in those made of lipid alone or with proteolipid of peak 1 (Fig. 6).
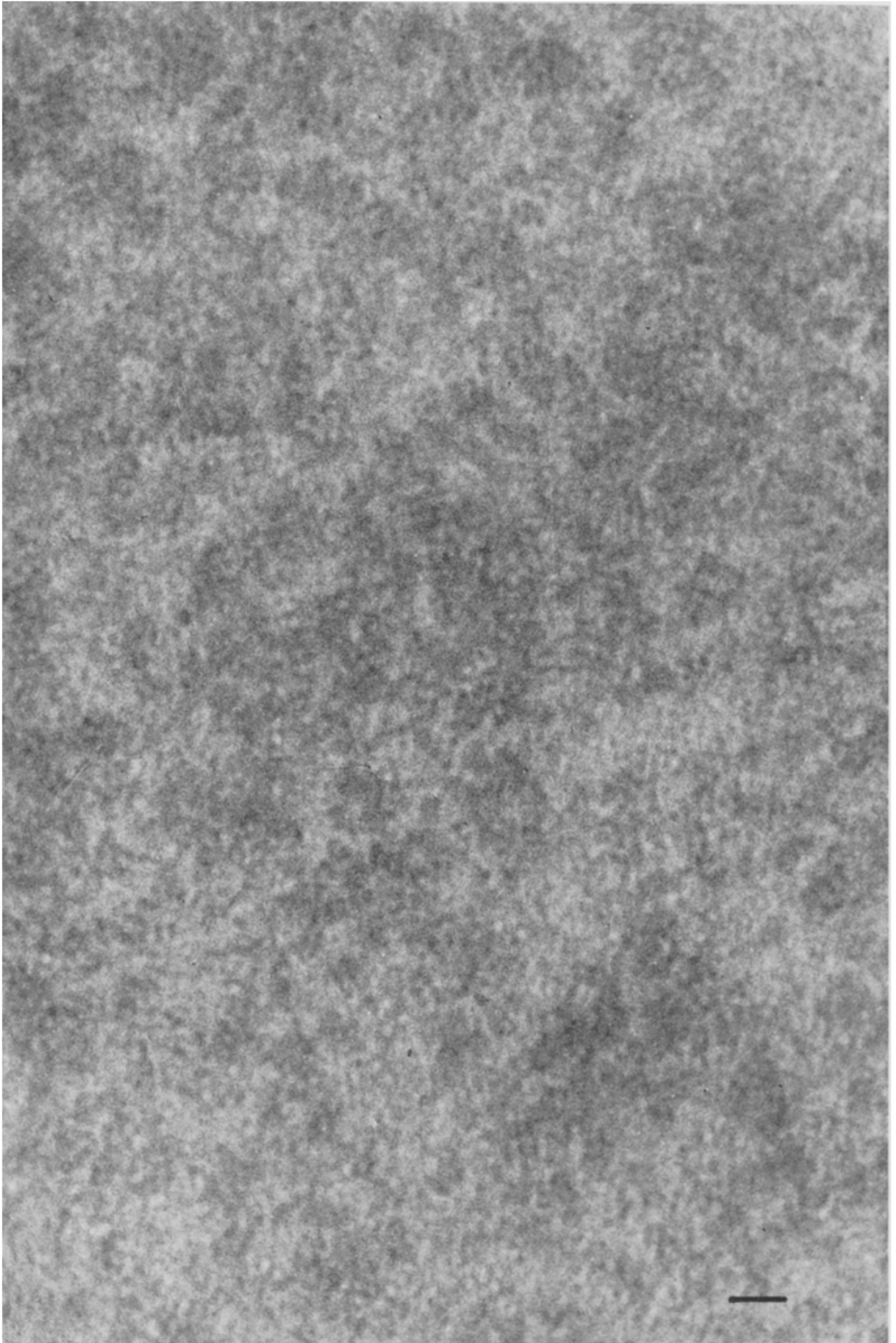
Fig. 2. Electron micrograph of a lipidic artificial membrane transferred onto an empty grid. Zones with different electron density are observed. In spite of the low magnification, the clear globular units later described in Fig. 3 made the bulk of this membrane. × 400,000
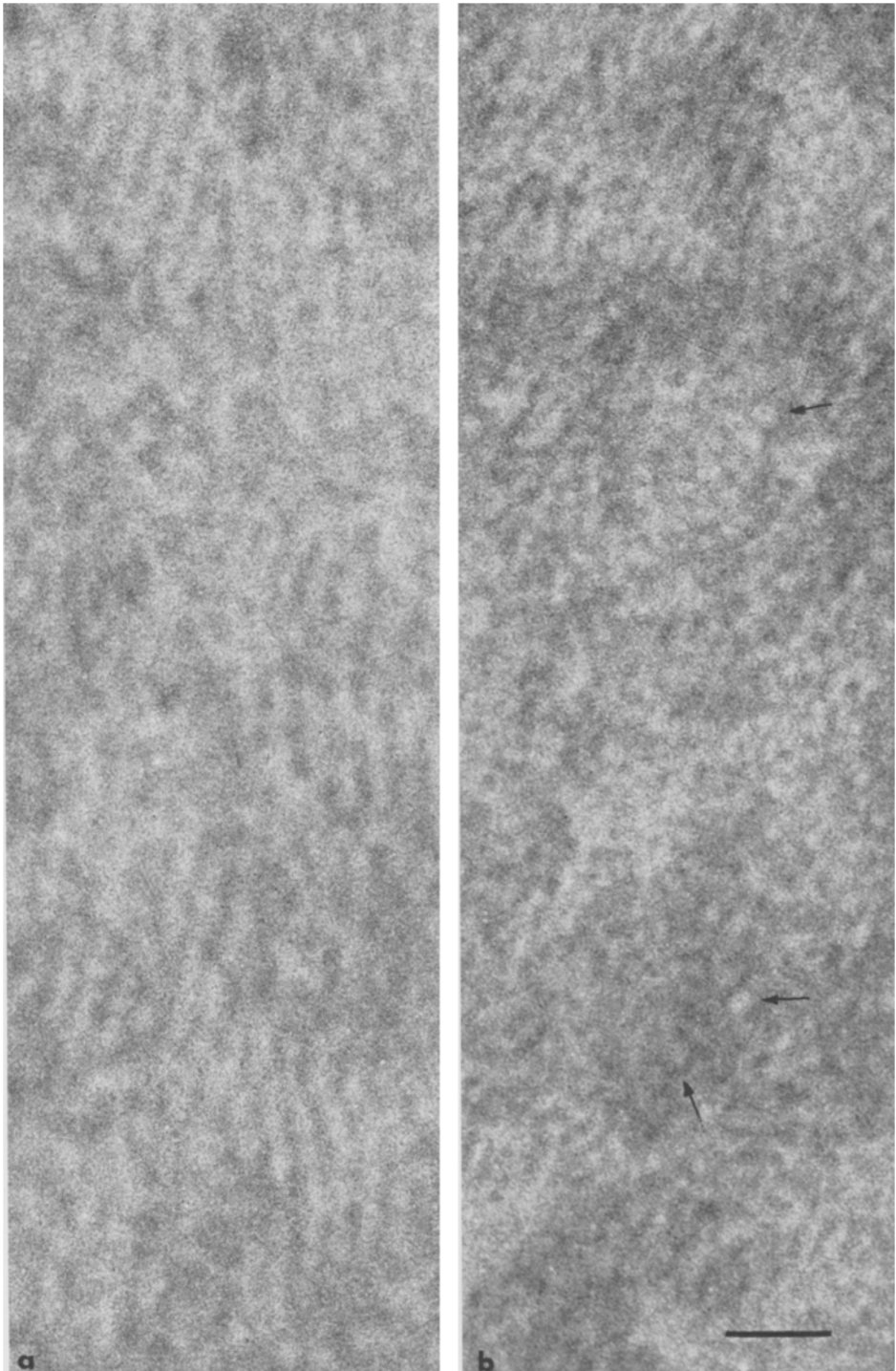
Fig. 3 a and b. High magnification view of a lipidic membrane after 2% glutaraldehyde fixation and treatment with osmium tetroxide vapors. (a) Periodic dense and clear bands are observed. (b) Globular elements (arrow) with a mean diameter at 40 Å are seen. The bar indicates 200 Å. ×720,000
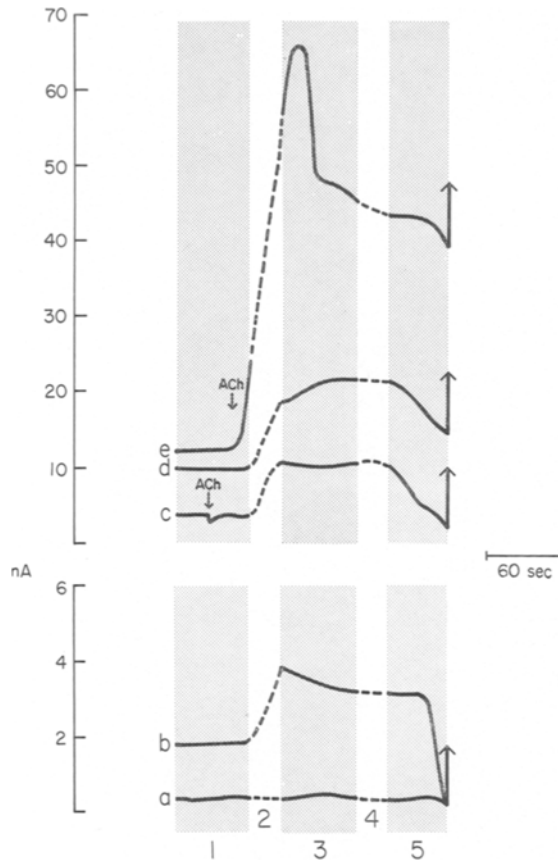
Fig. 4. Recording of current changes in artificial membranes in the course of the different periods of the experiments. In all cases the transmembrane potential is clamped at 100 mv. Numbers at the bottom of the figure indicate the following periods: 1) membrane current before fixation; 2) perfusion of glutaraldehyde into the chamber; 3) postfixation; 4) placing of the grid on the membrane; 5) sticking of membrane to the grid and final removal (arrows). (a) Membrane made with proteolipid of peak 3 without addition of phospholipids and cholesterol; the current is about 1 NA and does not change with glutaraldehyde fixation. (b) Membrane of brain phospholipids and cholesterol; current is about 2 NA and increases slightly with glutaraldehyde. (c) Membrane of brain phospholipids and cholesterol with addition of proteolipid of peak 1; see the lack of reaction to acetylcholine (ACh) and the slight increase with fixation. (d) Membrane of brain phospholipids and cholesterol with addition of proteolipid of peak 3. The effect of glutaraldehyde is of the same magnitude as in (c). (e) Membrane of brain phospholipids and cholesterol with the addition of proteolipid of peak 3; in this case ACh was added before fixation but the conductance change persisted during the fixation period. Observe that the final current change is much higher than in (d)
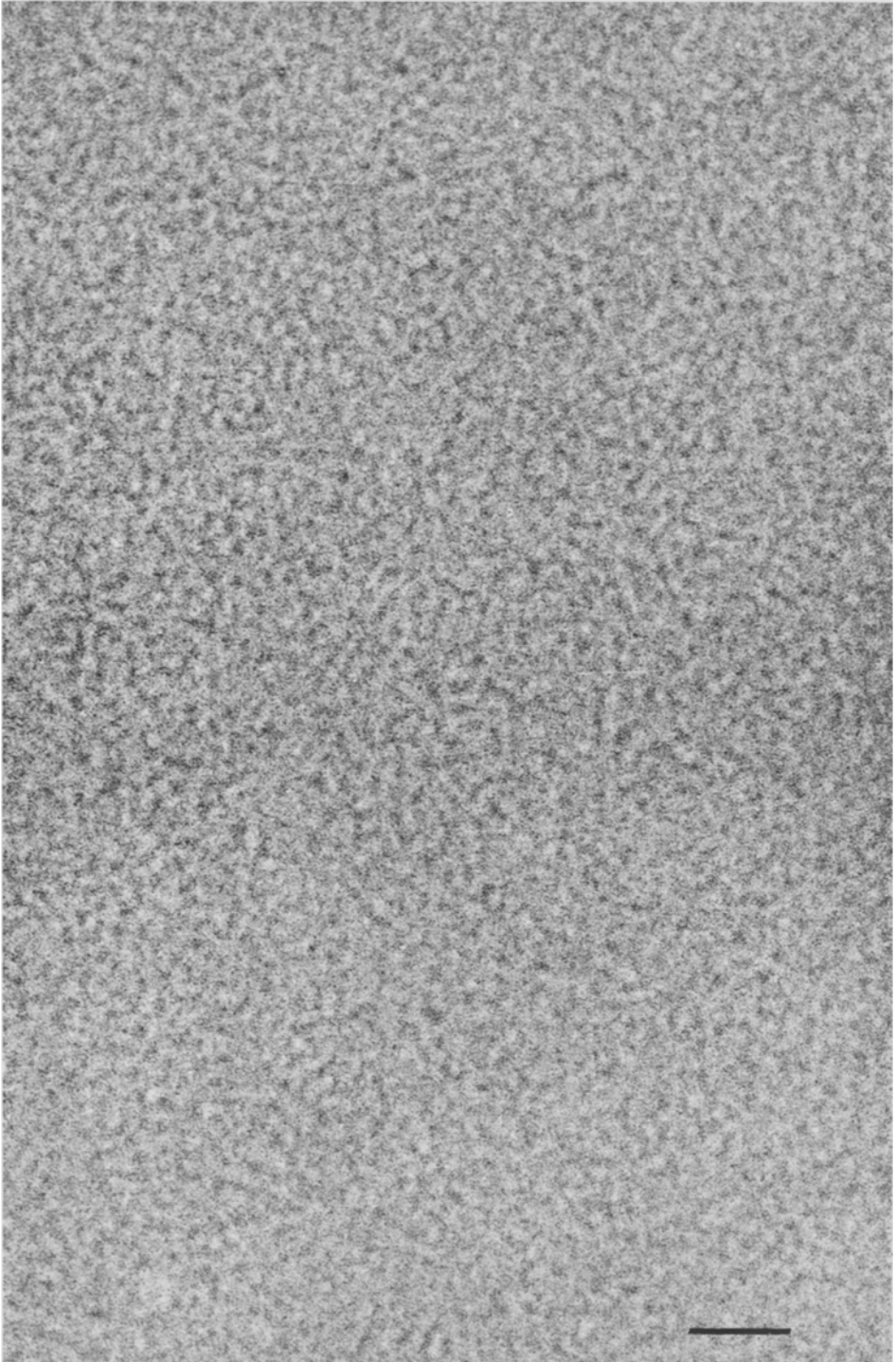
Fig. 5. High magnification of a control lipidic membrane containing proteolipid of peak 3 after glutaraldehyde fixation and treatment with osmium tetroxide vapors. The structure is rather smooth and uniform. Its texture should be compared with that of Figs. 3 and 7. The bar indicates 200 Å. ×720,000
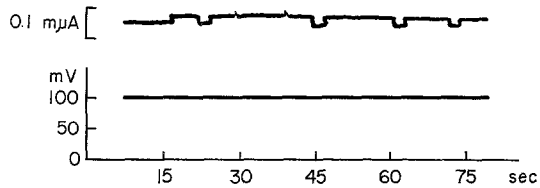
Fig. 6. Discrete current jumps observed in membranes with proteolipid from peak 3. The top trace shows current, the bottom trace shows applied voltage

Effect of Acetylcholine

The injection of acetylcholine on the membranes made of lipid and proteolipid from peak 1 produced practically no change in conductance (Fig. 4c) and, under such conditions, these membranes showed no changes in their ultrastructural pattern as compared to the controls.

When acetylcholine was injected upon membranes containing lipids and proteolipids from peak 3 ("receptor peak") a sudden fivefold increase in membrane conductance of a transient nature was observed (Fig. 4e). The electron micrographs revealed two distinct patterns. When the membranes were taken immediately after the acetylcholine addition and during the phase of increase in d–c, the membranes showed more contrast and had a more corrugated structure, with randomly dispersed dark spots having a mean diameter of about 20 Å (Fig. 7). Such spots generally showed a denser center and were the most conspicuous change observed in these acetylcholine activated membranes.

When the membranes were recovered some time after the acetylcholine injection or when the response was fading, the electron-microscope appearance was found to be similar to that of the controls (see Fig. 5), suggesting that the change in ultrastructure was also transient. The addition of glutaraldehyde to the medium, immediately after the acetylcholine injection, did not block the transient reaction. When the membrane was fixed with glutaraldehyde, prior to the injection of acetylcholine, no electrical response was elicited and the membrane ultrastructure was similar to that of the controls showing a rather smooth aspect without the dark spots.

Proteolipidic Membranes

Artificial membranes were also made with proteolipid of peak 3 alone, without the addition of phospholipids from gray matter and cholesterol. These membranes were thin, smooth and showed little contrast with osmium
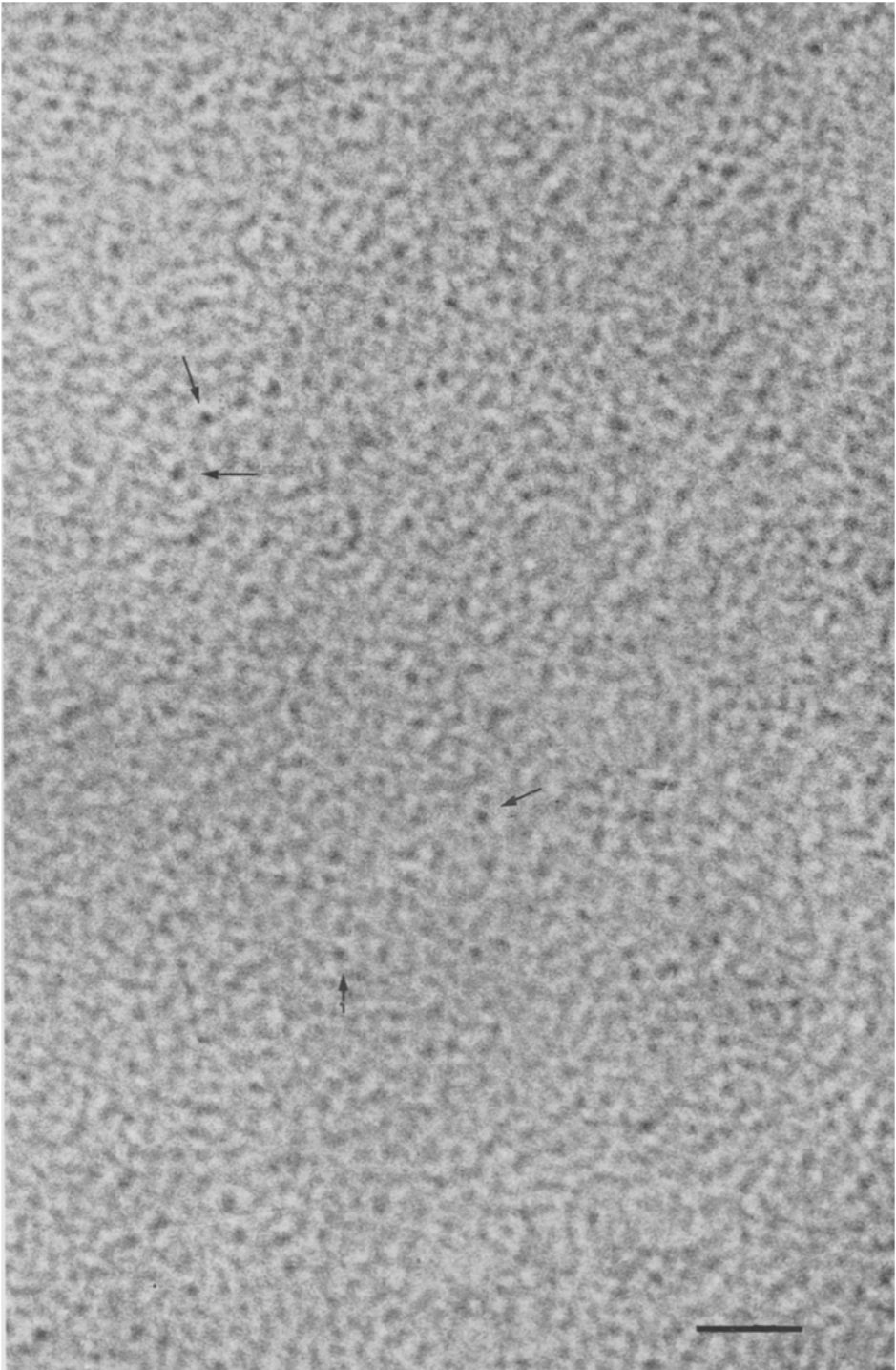
Fig. 7. High magnification of a lipidic-proteolipid (peak 3) membrane treated with acetylcholine. Glutaraldehyde fixation was done at the peak of the conductance change. The membrane fine structure has changed as compared with Fig. 5. This membrane shows more contrasted areas and a rather corrugated structure. Dense spots of about 20 Å with a darker center are observed. Arrows point to some of these spots. The bar indicates 200 Å. × 720,000

tetroxide. They were very fragile and unstable in spite of the glutaraldehyde fixation. The electrical resistance was about $1.0 \times 10^6 \ \Omega cm^2$, the highest in this series of membranes (Fig. 4a). No conductance response was obtained with the addition of acetylcholine and no changes in surface structure were observed.

Discussion

Fine Structure of Artificial Membranes

To our knowledge there are no reports in the literature of studies on the planar fine structure of artificial membranes made between two liquid media. In the present work an adequate fixation and a special technique for transferring the membrane onto supporting carbon films or onto empty grids has permitted us to obtain structure stable enough to be observed directly under the electron-microscope. The membranes were prepared while submitted to a fixed voltage and to different experimental conditions. The first questions we asked ourselves were whether the artificial membranes were really transferred onto the grids and, if this was the case, whether the observed structure was not influenced by that of the carbon support. Both uncertainties were ruled out with the use of empty grids to hold the artificial membranes. We observed that within the holes of those grids there was a definite structure corresponding to the artificial membrane. This was particularly evident in those membranes in which a break was produced showing definite edges at the empty space. Since the fine structure of the lipidic membranes observed without supporting film (Fig. 2) was identical to those transferred onto carbon films (Fig. 3a and b) we could be confident that this support did not introduce a heavy background noise that could interfere with the structure of the artificial membranes.

Without staining, the membranes were barely visible, and staining with osmium tetroxide vapors was indispensable to study its fine structure. Fixation in glutaraldehyde probably did not introduce drastic ultrastructural changes in the lipoprotein organization as the membrane conductance was only slightly modified by this fixative.

Membranes made of total phospholipids from cerebral cortex and cholesterol showed a special ordered structure in which clear globules of about 40 Å in diameter surrounded by a more dense diffuse matrix were prominent. These globular structures may be related to those described by Lucy and Glauert (1964) and Lucy (1968) in negatively stained preparations of lecithin and cholesterol in water which they interpreted as lipid micelles

with the polar groups toward the surface. In our experiments, lipids were originally in an organic solvent and during the organization of the membrane the orientation of the dipolar molecules within the micelle could be different than in water.

Membranes of a similar chemical composition but having a minimal amount of proteolipid, either of peak 1 or 3 from *E. electricus*, showed a much smaller electrical resistance, a lower electron density and a smoother texture in which no globular elements or bands could be detected. The presence of the hydrophobic protein has apparently led to a rearrangement of the lipidic structure.

The artificial membranes made of proteolipids from *Electrophorus* without the addition of lipids showed very special characteristics. They were very thin, had an amorphous appearance and an unusually high electrical resistance. Since in peak 3 the lipid/proteolipid ratio was about 4 (w/w) these membranes always contained a certain amount of lipid.

Ultrastructural and Conductance Changes
Observed under the Action of Acetylcholine

Previous work from this laboratory had shown that the so-called receptor proteolipids appeared under the electron-microscope as rod-shaped filaments that could undergo organization into parallel assemblies by the action of several neuroactive drugs: i.e., atropine sulfate in the case of the brain proteolipid (Vásquez, Barrantes, La Torre & De Robertis, 1970); acetylcholine and hexamethonium for the proteolipid of *Electrophorus* (Barrantes, Vásquez, Lunt, La Torre & De Robertis, *unpublished*). These findings suggested that these macromolecules were able to interact under the influence of the bound ligand. The previous observations of Parisi *et al.* (1971) that acetylcholine produced transient conductance changes in the lipidic membranes containing the proteolipid of peak 3 from *Electrophorus* and that it had no effect on those containing the proteolipid from peak 1 were confirmed here. In this last type of membrane, no ultrastructural changes were observed under the action of acetylcholine and its appearance was similar to that of control membranes. On the contrary, well-defined ultrastructural changes were observed in those lipid-proteolipid membranes that did react to acetylcholine.

In membranes fixed during the transient increase in conductance the smooth and compact fine structure of the control was replaced by a more uneven or "corrugated" appearance. In addition to this transformation the most striking change caused by acetylcholine was the appearance of

dense spots of about 20 Å in diameter with a more stained center distributed at random on the membrane. All these changes imply an increase in contrast (i.e., of osmium binding) in these membranes. It is interesting to emphasize that the conductance and structural changes due to acetylcholine were prevented by the prior fixation of the membrane with glutaraldehyde. On the contrary, when the conductance change was initiated it was not stopped by the fixative.

Two different mechanisms have been proposed to explain the permeability changes induced by certain types of molecules, especially the macrocyclic antibiotics, when added to artificial lipid membranes: i.e., the so-called carrier and the channel hypothesis. We have no direct information about the mechanism by which the receptor proteolipid molecule acts, but it is tempting to correlate the presence of the dense spots in the membrane with the transient permeability changes that take place under the action of acetylcholine. Such an idea is reinforced by the fact that upon the vanishing of the conductance change the membrane fine structure apparently reverts to the control conditions.

The number of dense spots measured in several electron micrographs was of the order of $3,500 \pm 100$ per μ^2 in the case of membranes fixed at the maximum of the conductance change by acetylcholine. On the other hand, the number of ions per μ^2 and per sec which, according to the current change, should traverse the membrane is of about 120,000. The size of the dense spots is of the same order of magnitude as the channel size calculated for solutes in different cell membranes (*see* Dowben, 1969).

An additional support for the channel hypothesis is found in the discrete fluctuations of conductance that may be observed in lipidic membranes containing proteolipid of peak 3 (Fig. 6). Similar current jumps were previously observed by Ehrenstein *et al.* (1970) in membranes containing excitability inducing material (EIM) and were interpreted as results of the opening and closing of a few channels in the membranes.

Recent studies by De Robertis, Lunt and La Torre (1971) have shown that there probably is a single high affinity binding site for acetylcholine in each proteolipid molecule of peak 3 from *Electrophorus*. In the case of the artificial membranes made exclusively of this proteolipid, it seems justifiable to assume that they have also bound acetylcholine; however, in this case both the conductance and ultrastructural changes were not present. We may speculate that a certain amount of lipid in the membrane is indispensable for the ionic translocation to occur.

In the drug-receptor interaction we may differentiate the binding and the ionophoric response as two different but coordinated functions. De

Robertis (1971) has postulated a macromolecular model for the cholinergic receptor in which several rod-shaped proteolipid molecules in parallel traverse the thickness of the lipid bilayer. In this model the receptor site would be in the outer end of each proteolipid molecule while the rest of it could represent the ionophoric region or potential channel. The opening of such a channel, with the subsequent ionic translocation, could result in a decrease in the forces holding together the individual proteolipid molecules. It is also possible that a certain lipidic milieu, surrounding the proteolipid molecules, is necessary to facilitate their displacement and the translocation of ions through the membrane. This type of model gives a logical explanation for the results presented here but other possible interpretations can not be excluded.

Several properties of artificial membranes are similar to those of biological membranes. The visualization under the electron-microscope of transient changes in the membrane fine structure in parallel with the conductance changes induced by acetylcholine may be interpreted as a manifestation of the "chemical excitability" of this membrane containing the cholinergic receptor.

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