LIPID BILAYERS AND LIPOSOMES IN RECONSTITUTION EXPERIMENTS WITH CHOLINERGIC PROTEOLIPID FROM TORPEDO ELECTROPLAX

P. Schlieper and E. De Robertis
Instituto de Biología Celular, Facultad de Medicina, Universidad de Buenos Aires, Buenos Aires, Argentina

Received February 23,1977

SUMMARY: The cholinergic proteolipid from Torpedo electroplax was used in reconstitution experiments in artificial membranes being incorporated directly into the membrane-forming solution or into liposomes (proteoliposomes) which interacted with lecithin bilayers. In both cases the membrane became reactive to acetylcholine by a decrease in resistance and an increase in the frequency and amplitude of minute current fluctuations of $3 \cdot 10^{-11}$ to $4 \cdot 10^{-10}$ mho. The injection of d-tubocurarine produced an increase in membrane resistance and a decrease in the amplitude of the current fluctuations. These results suggest that the cholinergic proteolipid is reconstituted in an active form in the bilayer.

INTRODUCTION Membrane model systems are being widely used in reconstitution experiments for a variety of proteins having special functions (1).

Several attempts have been made to incorporate detergent-extracted cholinergic receptor proteins from electroplax or brain into lipid vesicles (2,3) or planar membranes (4-6). The results are, so far, scanty and in most cases the systems were unreactive to the application of cholinergic drugs (5,7,8). One of the main difficulties in this work resides in the complete elimination of the detergent and in its possible effects on the membrane; recently we have found that Triton X-100 produces channels across artificial membranes (9). Furthermore since these hydrophobic proteins have to be added from the water phase, their integration into the bilayer may be incomplete (10). Some of these problems are avoided using proteolipids (11) for the reconstitution

ISSN 0006-291X

experiments. Proteolipids from the electroplax of Electrophorus or Torpedo, which bind cholinergic drugs, can be purified either by Sephadex LH-20 (12,13) or by affinity chromatography (14). Since they are dissolved in organic solvents they can be incorporated directly into the membrane-forming solutions used to make bilayers or phospholipid vesicles (i.e. liposomes). Previous experiments with a proteolipid from Electrophorus, showed transient changes in conductance (15-17) and fine structure (18) in artificial membranes by cholinergic drugs.Because these membranes were thick and of low resistance (ca:$2 \cdot 10^5$ ohm cm^2) they did not allow the study of the fine current fluctuations that will be described here and which may be regarded as "membrane noise". Here the cholinergic proteolipid from Torpedo was incorporated into artificial membranes by two different methods: a) dissolved into the membrane-forming solution,b) as proteoliposomes brought into contact with bilayers of pure lecithin. In both systems changes in conductance and "membrane noise" were observed under the action of acetylcholine and d-tubocurarine.

MATERIAL AND METHODS: Preparation of the cholinergic proteolipid from Torpedo:
Frozen electroplax of Torpedo Marmorata were thawed and homogenized in distilled water to give a final homogenate of 10% (w/v), which was frozen and lyophilized.From aliquots of 500mg of the dried tissue the cholinergic proteolipid was extracted with chloroform-methanol (2:1, v/v) as previously described (13) and separated by: a) Sephadex LH-20 chromatography (13) and b) affinity chromatography (14). In this case the desorption was done by a pulse of acidified chloroform-methanol (2:1 or 1:1, v/v; o.1 N HCl). The cholinergic proteolipid obtained was diluted with chloroform and evaporated several times under vacuum and N_2 to eliminate the acid. From an extract of 500 mg of dried tissue (2.5 g fresh tissue), approximately 280 µg of proteolipid was obtained.
Preparation of the black lipid membranes.The forming solution of control membranes consisted of 2% purified egg lecithin in decane (Sigma). For the experimental membranes minute amounts of the cholinergic proteolipid (4-30 µg/ml) were

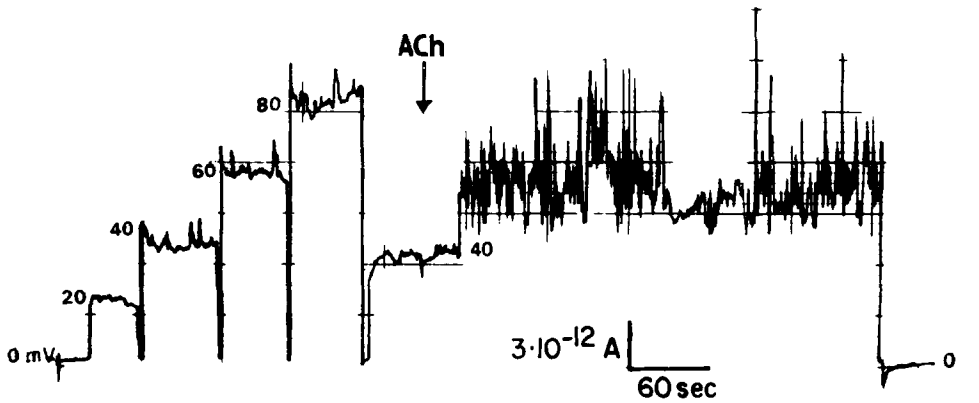

Fig.1. Time-current trace of a black lipid membrane made with 2% egg lecithin in decane containing 4 µg/ml of cholinergic proteolipid protein from Torpedo purified by Sephadex LH-20 chromatography. In both chambers the solutions contained 1 M NaCl in 1 mM Tris-PO_4 buffer pH 7.5. At the time indicated 10µl of 10^{-2}M acetylcholine was applied.

added to this solution. Bilayers were made in a horizontal position by applying about 1 µl on the 0.9 mm hole in a teflon septum, separating two aqueous chambers which contained the same saline solution buffered with Tris PO_4.The drugs, dissolved in the same solution, were microinjected near the upper side of the membrane. Since the dilution in the upper chamber is 1:1000, at equilibrium, the concentration of the drug is reduced by that factor. Ag-AgCl electrodes were used to clamp various voltages across the membrane. The current was measured by means of a Keithley 610 C microammeter and recorded with a Keithley inscriptor (Model 370). A two channel storage oscilloscope (Tektronix 5103 N)connected to a 1029 Philbrick preamplifier, through one channel, allowed the recording of the voltage applied and the membrane potential. The other channel was connected to the output of the ammeter to record the fast current changes. Control membranes showed resistances varying between 1.6 to $6 \cdot 10^7$ ohm cm^2.

Preparation of liposomes and proteoliposomes: Lipid vesicles were made either with egg lecithin (liposomes) or egg lecithin with the cholinergic proteolipid separated by affinity chromatography (proteoliposomes).In the latter case 200 ug of proteolipid was mixed with 1.5 µmol of egg lecithin and 0.1 µmol of phosphatidic acid in chloroform. The solvent was then evaporated by a jet of N_2 followed by vacuum for 15 min. Then 1 ml of saline solution was added and the samples were sonified in the water bath of a Kerry sonifier at 50 Kc/s for 30 minutes. The resulting suspension was transparent and bluish and under the electronmicroscope mostly unilamellar vesicles were observed.

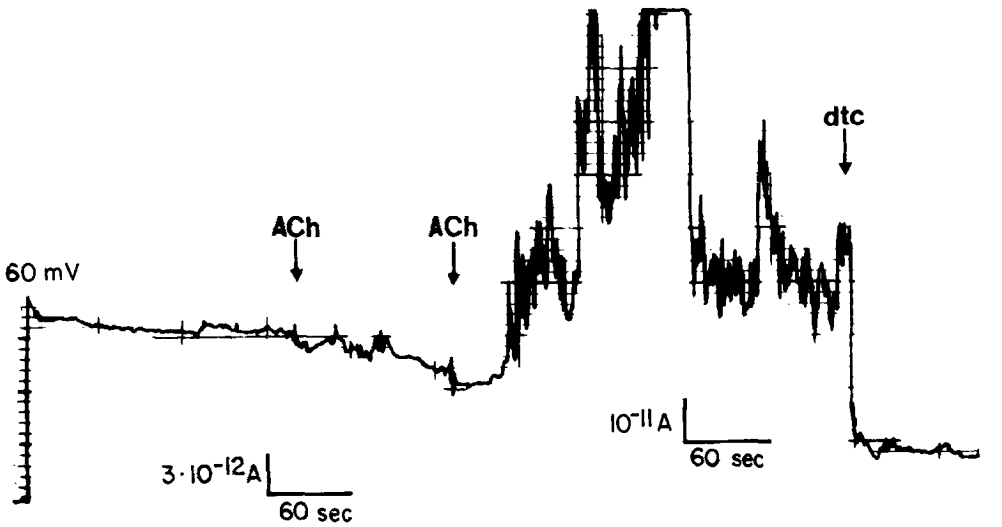

Fig.2. The same as in Fig.1 but from a membrane containing 30µg/ml of cholinergic proteolipid from Torpedo purified by affinity chromatography. In both chambers the solutions were 0.1M NaCl in 1 mM Tris-PO_4 buffer pH 7.3. At the times indicated 10µl of 10^{-2}M acetylcholine or of 10^{-3}M d-tubocurarine were applied.

RESULTS

Action of acetylcholine and d-tubocurarine on proteolipid containing bilayers.

Fig. 1 shows a black lipid membrane clamped at voltages (V) varying stepwise between 20 and 80 mV.An ohmic relationship of the current (I) versus V is observed.Already at 20 mV there are small fluctuations of I that, with higher potentials, increase in amplitude but not in frequency.30 seconds after a single injection of acetylcholine at 40 mV there is a sudden current jump into a higher level of conductance ($\Delta G=1.3 \cdot 10^{-10}$ mho), which is accompanied by a discharge of fluctuations of higher amplitude and frequency, for several minutes, until the voltage is dropped to zero. The maximum amplitude of the jumps observed before the application of the drug is about 10^{-12}A ($\Delta G=2$ to $3 \cdot 10^{-11}$mho). After application of acetylcholine

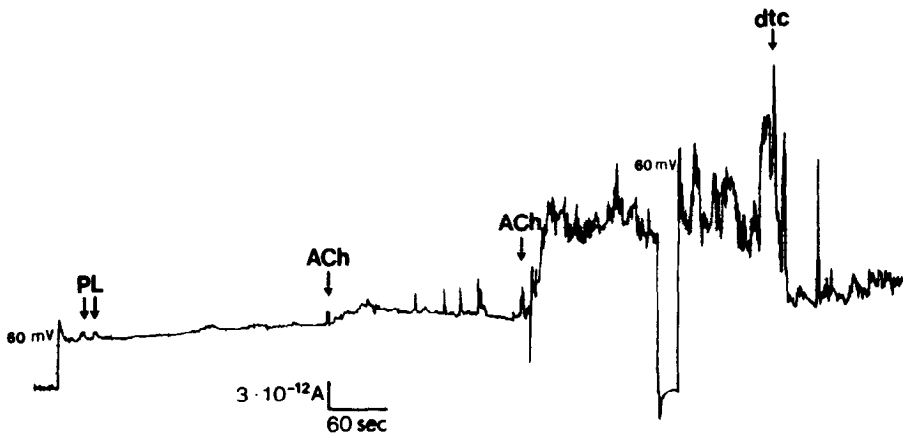

Fig.3. Time-current trace of a membrane made with 2% egg lecithin in decane. The bathing solution was 0.1M NaCl in 1 mM Tris-PO_4 buffer pH 7.3. At the times indicated,5 µl of proteoliposomes (PL), containing the cholinergic proteolipid from Torpedo, and 10 µl of 10^{-2} M acetylcholine or of 10^{-3} M d-tubocurarine were applied.

the maximal fluctuations increase considerably ($1.6 \cdot 10^{-11}$A, $\Delta G = 4.0 \cdot 10^{-10}$mho). The membrane resistance (R) is reduced from $1.5 \cdot 10^7$ ohm cm^2 to $9.0 \cdot 10^6$ ohm cm^2. The average frequency of the fluctuations is about 13/min before and 53/min after the application of the acetylcholine.

In Fig. 2 the proteolipid containing membrane was constantly clamped at 60 mV. Two injections of acetylcholine induced a rapid increase in G and in the amplitude and frequency of the fluctuations; R decreased from $1.5 \cdot 10^7$ ohm cm^2 to $2 \cdot 10^6$ohm cm^2 and the maximal fluctuations were of the order of $1.2 \cdot 10^{-11}$A. An injection of d-tubocurarine caused a reversal of the effect of acetylcholine upon the membrane. I and R,as well as the amplitude of the fluctuations returned to the same level as in the control state. The effect of d-tubocurarine was persistent and, in this case, blocked the action of two more injections of acetylcholine; however, in other experiments the effect of d-

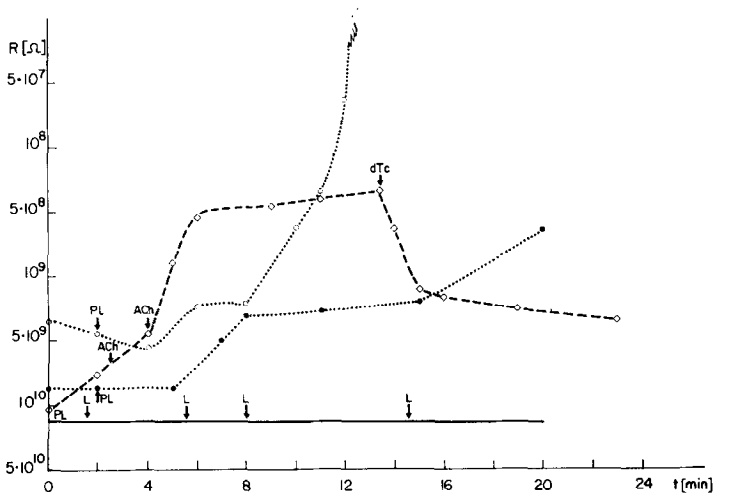

Fig.4. Plot of resistance versus time of four different lecithin membranes in the presence of liposomes (L) or proteoliposomes (PL). The dashed curve represents an experiment in which the membrane was made in the presence of 10µl of proteoliposomes. Each arrow indicates an amount of 10µl of the corresponding substance, added to the bathing solution.

tubocurarine could be overcome by more injections of acetylcholine.Effects essentially similar to those described here were also observed in membranes which were bathed in KCl 0.1M, Tris-PO_4 buffer at pH 7.3. Control membranes did not show current fluctuations after acetylcholine.

Action of liposomes and proteoliposomes on lipid bilayers. In a second series of experiments, proteoliposomes,prepared as indicated in methods, were brought into contact with the lecithin membrane. They produced two interrelated changes in the membrane properties: a)appearance of small fluctuations, b)changes in membrane conductance. Both effects were influenced by cholinergic drugs. Fig. 3 shows an example of these type of changes. A total of 10 µl of proteoliposomes were applied on a membrane clamped at 60 mV; after acetylcholine small fluctuations appeared which, after a second injection, increased in amplitude

and frequency. The resistance of the membrane decreased from $2.3 \cdot 10^7$ to $9 \cdot 10^6$ ohm cm^2. d-Tubocurarine reduced the conductance of the membrane and the amplitude of the fluctuations.

Fig. 4 shows four different experiments in which pure lipid liposomes (L) or proteoliposomes (PL) were injected. In the experiment shown by a solid line four injections of pure liposomes did not change the resistance of the membrane. In another experiment (●—●), a single injection of proteoliposomes produced a marked decrease in resistance which, starting from $9 \cdot 10^9$ ohm reached $6 \cdot 10^8$ ohm after 20 min. In a similar experiment(o·····o) the membrane underwent a considerable decrease in R until it broke. In the experiment indicated by the dashed line (□- -□)the decrease in resistance became much more rapid after acetylcholine and this was reversed by d-tubocurarine.

DISCUSSION

Katz and Miledi (19) defined as "acetylcholine noise"the minute fluctuations in membrane voltage (0.22 μV) that were produced in the frog end plate, depolarized by acetylcholine. Anderson and Stevens (20) with voltage clamping found fluctuations of $\Delta G = 3 \cdot 10^{11}$mho ($2 \cdot 10^7$ ions/sec).

In the present work the incorporation of the proteolipid into the black lipid membranes produced current fluctuations of the same order of magnitude, which increased in amplitude and frequency with acetylcholine and were reduced after d-tubocurarine. The main difference between this noise and that of the end plate (20) is the duration of a single event, which in our case is roughly 100 times longer. However the resistances of our membranes were four orders of magnitude higher. From the average fluctuations induced by acetylcholine we have calculated an ion flux of $3 \cdot 10^7$ ions/sec which is in the range of that reported for

the end plate, but it is five orders of magnitude higher than that published by Kasai and Changeux (21) in membrane microsacs of Electrophorus. The results presented here suggest,that the cholinergic proteolipid from Torpedo may be reconstituted in the artificial membrane in a form that is "reactive" to cholinergic drugs. A more detailed and statistical analysis of the observed membrane noise could not be made till now, but further studies are in progress.

ACKNOWLEDGMENTS

The excellent collaborationof Dr. María C.L. de Carlin and Mrs. Lina L. de Stein in the isolation of the proteolipids and that of Dr. E. Ochoa in the preparation of the liposomes is acknowledged.
This paper was supported by grants from the Burrows Wellcome Laboratory (USA) and the CONICET (Argentina). Dr.Peter Schlieper is a fellow of the Deutsche Forschungsgemeinschaft.

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