Acetylcholine-Induced Excitation on Bilayers

When some years ago MÜLLER and RUDIN[1] introduced the technique of forming stable lipid bilayer membranes between two aqueous phases, it soon became evident that such membranes have a structure closely similar to the bimolecular lipid lamella which DANIELLI and DAVSON[2] had proposed as the structural core of biological membranes. Moreover some physical properties of bilayers, such as permeability to water, small-molecule permeability, surface tension and capacitance, to name just a few, correspond closely to values exhibited by biological membranes. But even though the general correspondence between the physical properties of the two systems is striking, the permeability and selectivity of the bilayers to ions is quite different. And yet these are exactly the properties which are important for the most interesting physiological membrane processes such as active transport and electrical excitability.

From a biochemical point of view, also, it is evident today that properties like selectivity cannot be derived from simple lipid bilayers but must come from more complex structures also containing proteins. It is therefore of considerable interest that a number of cyclic polypeptides have been shown to induce in lipid bilayer membranes not only low resistance but also a cation selectivity which closely parallels that found in biological membranes[3]. For instance, valinomycin, a cyclic dodecadepsipeptide, at concentrations as low as 10^{-7} g/ml lowers the electrical resistance of the bilayer to about $10^3\ \Omega\ cm^2$. For this experiment the permeability series for H^+ and the alkali metal ions has been reported to be $P_{H+} > P_{Rb+} > P_{K+} > P_{Na+} \simeq P_{Li+}$. This is of the order of permeability most often observed in natural membranes.

DEL CASTILLO[4] went a step further, and modified the lipid bilayers by adding either antigens or enzyme to them. He then observed impedance changes by afterwards adding antibodies or substrate to the modified membranes. He was able to demonstrate convincingly that the impedance changes are due to the formation of antigen-antibody or enzymesubstrate complexes at the film-water interphase. One can assume that the sudden drop in film impedance is caused by the steric changes that antibody or enzyme molecules undergo when they interact with the specific antigens or substrate.

Some physical properties of acetylcholinesterase (A-Chase)[5,6] support the hypothesis that the cholinergic receptor could be part of the macromolecular complex, which has been isolated from electrophorus electricus and shows the highest specific activity of AChase[7]. It is assumed that the cholinergic receptor undergoes conformational changes during activity and thereby produces in the excitable membrane a change in permeability to K and Na ions[8].

It was of great interest to see if one could also observe any change in the permeability of lipid bilayers to ions by incorporating AChase into the artificial membranes. We therefore repeated the experiments of DEL CASTILLO[9] and modified the bilayers by absorbing AChase of different specific activity (80; 310 and 710 mmoles acetylcholine hydrolyzed/mg protein/h) on to the membrane already formed, or by adding the enzyme to the aqueous phase prior to membrane formation. The symmetry conditions of the formed lipid-protein membranes were also investigated.

Materials and methods. Ox brains were chosen as a source of lipids, and were obtained immediately after slaughter. The white matter was removed, cooled to liquid nitrogen temperature, crushed and freeze-dried. 100 mg of the powder obtained is extracted with 5 ml of a 2:1 chloroform-methanol solution. After the addition of 0.5 ml NaCl 0.1 *M*, the solution is centrifuged and the upper phase discarded. 0.15 ml of the lower phase is evaporated to dryness and dissolved again in 0.5 ml of 2:1 chloroform-methanol. 75 mg α-tocopherol (purified by chromatography) and 15 mg of recrystallized cholesterol is added to the final lipid solution. With this solution the bilayer formation is carried out according to MÜLLER and RUDIN (see Figure 1 for the arrangement) and the resistance determined by measuring the potential drop across the lipid membrane. The enzyme is added either to the aqueous phase before the bilayer is formed or else added to the aqueous phase on one side after formation, after which some minutes should be allowed in order to permit a certain interaction with the bilayers. Acetylcholinesterase purified according to LEUZINGER et al.[7] with different specific activities has been used.

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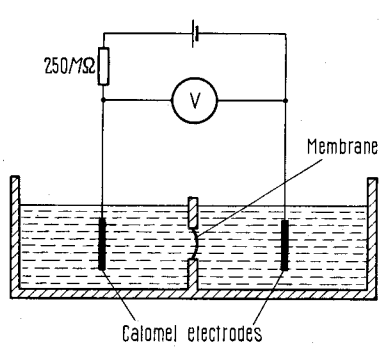

Fig. 1. Experimental arrangement for electrical measurements.

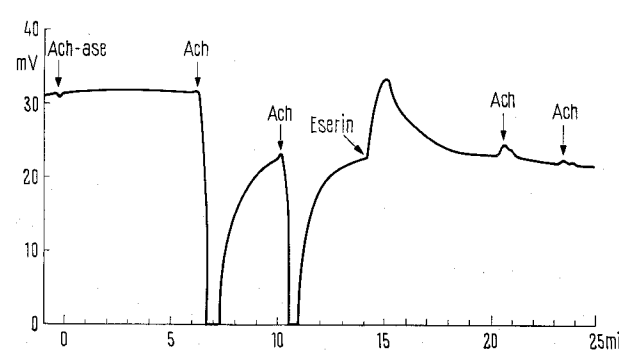

Fig. 2. Records of potential difference across lipid bilayers containing acetylcholinesterase.

Results. Figure 2 shows a typical experiment. The starting resistance of the film was 100 $M\ \Omega\ cm^2$. As indicated in the figure, the addition of enzyme to the aqueous phase does not change the resistance, but as soon as acetylcholine (ACh) is added to the aqueous phase a drastic change can be observed, the resistance dropping by two orders of magnitude. As the substrate is hydrolyzed, the resistance increases slowly again to almost the same magnitude as before. This effect may be obtained over and over again. The limiting factor is the fragility of the membranes.

As a control, the enzyme was inhibited by eserine, so that it was prevented from forming the enzyme-substrate complex. In this case the bilayer becomes insensitive to acetylcholine (see Figure 2).

The experiment was also carried out with enzyme preparations of different specific activities. The extension of the observed effects – namely the number of coulombs passing through the membrane during impedance change – strictly dependent on the specific activity as long as the experimental conditions like protein concentration 1 µg/ml), pH, temperature and the amount of ACh added (5 µg/ml) have been kept constant, thus demonstrating that the change in permeability is closely related to AChase activity.

Another interesting result was the fact that adding the enzyme at one side of the already formed bilayer, then adding the substrate to the other side of the bilayer after 10 min, also produced a drop in resistance. This fact indicates that the enzyme is built into the membrane in such a way that the molecule is accessible from both sides and symmetrically oriented. In other words, these experiments suggest a mosaic structure for the lipid-protein membranes investigated similar to that which GREEN[10] proposes for the living cell.

Discussion. It is becoming more and more evident that the study of the behaviour of membrane-bound enzymes in solution can lead to misinterpretation of their mode of action. These enzymes do not exist in the membrane in a state of autonomy but form an indissociable continuum with the rest of the membrane, and it therefore seemed interesting to study AChase on a definite structure similar to the natural membrane, by incorporating the enzyme into bilayers. Though these bilayers naturally exhibit a much lower order of complexity than natural membranes, it can be assumed that they reflect a general design at least with regard to the basic structural elements of membranes.

The experiments demonstrated that bilayers containing a very small amount of AChase from electrophorus electricus reacted to the addition of ACh by a rapid and transient increase in conductance. In other words, a phenomenon was observed similar to that observable in excitable membranes.

We could further demonstrate the amount of coulombs passing through the membrane during impedance change definitely depends on the enzymatic activity present on the membrane. These findings demonstrate that AChase alone, together with its natural substrate ACh, is sufficient to produce a change in permeability to small ions in artificial membranes.

The assumption of a separate cholinergic receptor molecule independent of the $\alpha_2\ \beta_2$ complex of AChase seems to be unnecessary in these experiments. The data rather support a hypothesis[5] which assumes that α and β subunits of AChase fulfil different functions: the α-unit would represent the catalytic part and the β-unit the receptive part. The whole sequence of events leading to depolarization of the membrane would occur on the same complex, a condition which seems to be important with regard to the extreme rapidity of the processes. The individual steps would be as follows: the β-unit recognizes acetylcholine and interacts with it to form a complex which induces a conformational change that brings the catalytic site of the α-unit into close proximity with acetylcholine bound to the receptive site of the β-unit and is thereby in a position to hydrolyze ACh and reverse the whole effect.

Zusammenfassung. Es konnte gezeigt werden, dass Bilayers, die eine ganz geringe Menge Acetylcholinesterase enthalten, auf die Zugabe von Acetylcholine mit einer Änderung der Permeabilität gegenüber kleinen Ionen reagieren. Dieser Effekt ist reversibel und die Grösse des Effektes hängt bei konstanten Versuchsbedingungen nur mit der spezifischen Aktivität der Enzympräparation zusammen.

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Glycogen Content in Young and Old Rats Liver and Muscles

It was found that in muscles of aged rats the percentage of phosphocreatine to the total creatine is lower than in young animals[1]. Phosphocreatine is used for the rephosphorylation of ADP to ATP in the muscle. It was also found that by an increase of glucose feeding, the phosphocreatine values of old animals muscles return to those of young ones[2]. This may be related to changes in glycogen metabolism, and therefore the glycogen content of muscles and liver in young and old rats was compared.

Materials and methods. Wistar albino rats from the Institute's own colony were used. The preparation of muscles followed the former description[2]. The glycogen content was estimated by the method of VAN DER KLEIJ[3].

To test the influence of work, the animals were transferred into a narrow container with water at 25 °C[1]. After exhausting swimming, they were immediately killed and the glycogen content of liver and white and red muscles was estimated. The significance of the differences of results between young and old animals, and red and white muscles was checked by Student *t*-test.

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