Biochimica et Biophysica Acta, 506 (1978) 161—172
© Elsevier/North-Holland Biomedical Press

BBA 77895

INTERACTIONS OF ACETYLCHOLINE RECEPTOR AND ACETYLCHOLINESTERASE WITH LIPID MONOLAYERS

THERESE WIEDMER [a], URS BRODBECK [a,*], PETER ZAHLER [b] and BERNARD W. FULPIUS [c]

[a] *Medizinisch-chemisches Institut der Universität, Bühlstrasse 28, 3000 Bern 9,*
[b] *Biochemisches Institut, Freiestr. 3, 3000 Bern 9, and* [c] *Département de Biochimie, Sciences II, 1211 Genève 4, (Switzerland)*

(Received June 6th, 1977)

Summary

The interaction of acetylcholine receptor and acetylcholinesterase with lipid monolayers was followed by measuring changes in surface pressure.

When injected into the subphase of a lipid monolayer, the proteins caused increases in surface pressure from 5 to 10 dynes/cm, indicating a penetration of protein into the monolayer. At pH values below the isoelectric point of the proteins the incorporation was improved. The same was observed when Ca^{2+} (2 mM) was added.

The presence of the enzyme in the mixed film could be demonstrated by using diiso[^{3}H]propyl fluorophosphate-labelled acetylcholinesterase as well as by measuring enzyme activity. Acetylcholine receptor was shown to be present in the mixed film by using a complex made of the receptor and α-[^{3}H]neurotoxin.

Introduction

In the last few years several laboratories have focused their studies on the following question: is the acetylcholine receptor in excitable membranes identical to the ionic channel responsible for permeability changes, or is it only part of it? One way to approach this problem was to reconstitute an excitable membrane by incorporating the acetylcholine receptor into planar lipid bilayers and to measure the permeability changes after addition of a cholinergic agonist to the bathing solution. Such experiments were reported by different authors

Abbreviations: BBOT, 2,5-bis-[5'-*tert*-butylbenzoxazolyl-(2')]thiophene; iPr_2P-F, diisopropyl fluorophosphate; Nbs_2, 5,5'-dithiobis(2-nitro-benzoic acid); O-CH_3-phosphatidylcholine, 1,2-dipalmitoyl-3-*P*-methoxy-phosphatidylcholine.

* To whom correspondence should be addressed.

using acetylcholine receptor from several sources [1—5]. Another method was to incorporate receptors into liposomes loaded with $^{22}Na^+$ and to study the release of these ions upon addition of carbamylcholine [6]. The permeability changes observed in these two kinds of experiments would indicate that the reincorporated receptor displayed both the acetylcholine binding site and the molecular features for ion translocation. However, it should be noticed that a similar effect was reported by using acetylcholinesterase, a protein not supposed to contribute to ion movements [7—9]. A third method for the formation of bimolecular lipid membranes consists of combining two monolayers preformed at the air-water interface [10]. In this system the incorporation of a protein into one of the monolayers can be measured directly by monitoring the increase in surface pressure caused by the penetrating protein. Acetylcholine receptor [11—14] and acetylcholinesterase [15—17] can now be isolated in highly purified form. In order to gain further knowledge on the respective roles of acetylcholine receptor and acetylcholinesterase in inducing possible permeability changes it would be interesting to form bilayers according to that last method. These could contain acetylcholine receptor and/or acetylcholinesterase. In the present report we characterize the interaction of these proteins with monolayers made of different lipids in order to establish the optimal conditions for the formation of a bimolecular membrane exhibiting cholinergic properties. A preliminary account was presented elsewhere [18].

Materials and Methods

Materials

Trypsin (EC 3.4.21.4) (Type III from bovine pancreas) was purchased from Sigma Chemicals Co. (St. Louis, Mo., U.S.A.). Bovine serum albumin was from Poviet Producten (Amsterdam) and BBOT from Ciba-Geigy (Basel). Triton X-100 was from Rohm and Haas (Philadelphia), phenylmethylsulfonyl fluoride from Merck (Darmstadt), Nbs_2 from Fluka (Buchs). O-CH_3-phosphatidylcholine was obtained from Berchtold, Biochemisches Labor (Bern). Edrophonium chloride (3-hydroxyphenyldimethylethylammonium chloride) was a gift from Hoffmann-La Roche (Basel).

Methods

Preparation of acetylcholinesterases. Electric organ (100 g) of *Electrophorus electricus* was homogenized for 90 s in a VirTis 45 apparatus at 50% of the maximal speed in 200 ml of ice-cold buffer I (0.01 M sodium phosphate (pH 7.4)/1.0 M NaCl/0.001 M phenylmethylsulfonyl fluoride/0.05% (w/v) NaN_3). The resulting slurry was centrifuged (20 min, 19 000 $\times$ g) and the supernatant (220 ml) thoroughly mixed with toluene (110 ml). The phases were treated as described earlier [19]. The enzyme (20 000—60 000 units in 280 ml of buffer I) was added to 50 ml of an affinity gel prepared according to Ott et al. [20]. All the following steps were carried out at 4°C. After adsorbing the enzyme overnight, the gel was separated from the supernatant by centrifugation (30 min, 5000 $\times$ g) and washed in buffer I until the supernatant was colourless, usually 3—4 times. The gel was then packed into a column (2.3 20 cm) and rinsed for 4 days with buffer I. Acetylcholinesterase (EC 3.1.1.7)

was eluted with buffer II (0.01 M sodium phosphate (pH 7.4)/0.8 M NaCl/ 0.001 M phenylmethylsulfonyl fluoride/0.02 M edrophonium chloride and 0.05% (w/v) NaN_3). The enzyme eluted in the first 50 ml was dialysed against buffer I. The specific activity was 6000 I.U./mg protein. Acetylcholinesterase from human erythrocyte was purified as described by Ott et al. [20]. The specific activity was 3800 I.U./mg protein.

Enzyme assay. Acetylcholinesterase activity in solution was determined according to the method of Ellman et al. [21]. The assay mixture (3 ml) contained acetylthiocholine (1 mM), Nbs_2 (0.125 mM) and sodium phosphate, pH 7.4 (100 mM).

Radioactive labelling of acetylcholinesterase with diiso[^{3}H]propyl fluorophosphate. Acetylcholinesterase (490 I.U. in 180 μl buffer I) was mixed with 20 nmol of [^{3}H]iPr_2P-F in 20 μl of propylene glycol (final concentration 0.1 mM) and incubated at room temperature in a sealed recipient. Excess [^{3}H]-iPr_2P-F was removed by gel filtration on a Sephadex G-25 column (0.5×8 cm) equilibrated with buffer I without phenylmethylsulfonyl fluoride.

Preparation of acetylcholine receptor. Acetylcholine receptor from the electric organ of *Torpedo marmorata* was purified according to the method described by Klett et al. [11] for *E. electricus* with the following modifications: before elution of the receptor, the hydroxyapatite column was washed extensively (20 volumes) with a buffer containing 0.01% Triton X-100 instead of 1%. This low detergent concentration was also present in the elution buffer. The material desorbed from hydroxyapatite was subsequently dialysed overnight at 4°C against a buffer containing (0.1 M Tris · HCl (pH 7.4)/0.1 M NaCl and 10% (v/v) glycerol. Under these conditions the final Triton concentration was lower than 0.01%. A specific activity of 8 nmol ^{125}I-labelled α-bungarotoxin per mg protein was obtained.

Radioactive labelling of acetylcholine receptor with α-[^{3}H]neurotoxin. α-Toxin, purified from the venom of the cobra *Naja naja siamensis* was labelled with tritium according to the method described by Cooper and Reich [22]. In order to make the receptor-toxin complex, acetylcholine receptor was first incubated for 1 h with a 5-fold excess of α-[^{3}H]toxin. The unbound α-[^{3}H]-toxin was then separated by gel filtration using Sephadex G-100. The complex was adsorbed on a DEAE-cellulose column (2 ml) equilibrated with a buffer of potassium phosphate, pH 7.4 (0.01 M) containing 0.01% Triton X-100. The column was washed with 100 ml of the same buffer and the complex eluted with a buffer of potassium phosphate, pH 7.0 (0.05 M) containing 0.01% Triton X-100 and dialysed against 0.01 M Tris · HCl (pH 7.4)/0.1 M NaCl. Solutions containing 0.1 mg protein/ml and 56 400 cpm/ml were obtained.

Determination of protein. Protein concentration was estimated according to the method of Lowry et al. [23] using bovine serum albumin as standard.

Immobilization of trypsin. Trypsin was immobilized on Sepharose CL-4B using the buffer activation method described by Parikh et al. [24].

Isolation of lipids. Human erythrocyte ghosts were obtained according to Dodge et al. [25] and the total lipids extracted following the procedure of Folch et al. [26]. Sphingomyelin and phosphatidylcholine were isolated from the total lipid extracts as described by Kramer et al. [27]. Eel lipids present in 100 ml of toluene phase obtained during the preparation of acetylcholinesterase

were extracted twice with 200 ml of water. The mixture was centrifuged (7000 × *g*, 15 min) and the organic phase evaporated to dryness. The lipids were taken up in chloroform (2 mg/ml) and stored under nitrogen at −20°C.

Measurement of the surface pressure (monolayer technique). The surface pressure was determined by the Wilhelmy plate method as described by Morse [28]. The teflon trough of the Wilhelmy balance contained two compartments of approx. 7 ml each which were separated by a barrier. The lipid film spread over the surface of one liquid compartment could be transferred to the other by movement of that barrier. Thus the film could be exposed to the two subphases. For penetration studies the proteins were injected with a syringe into one of the subphases. The final protein concentration in the subphase was 3 μg/ml.

Results

When eel acetylcholinesterase or *Torpedo* acetylcholine receptor were injected into a subphase at pH 7 in the absence of a lipid film, a surface pressure of 17—19 dynes/cm was reached after 60 min. No differences were obtained for pH values of 3—7.

Penetration of acetylcholinesterase into lipid monolayers

When native eel acetylcholinesterase was injected into the subphase of a lipid monolayer made of erythrocyte lipids, a surface pressure of 24 dynes/cm was reached after 60 min. The surface pressure increase measured at different initial surface pressures and under different pH conditions is shown in Fig. 1. Even above the surface pressure of the protein alone (17—19 dynes/cm) an increase was observed. The total increases in the surface pressure were higher at

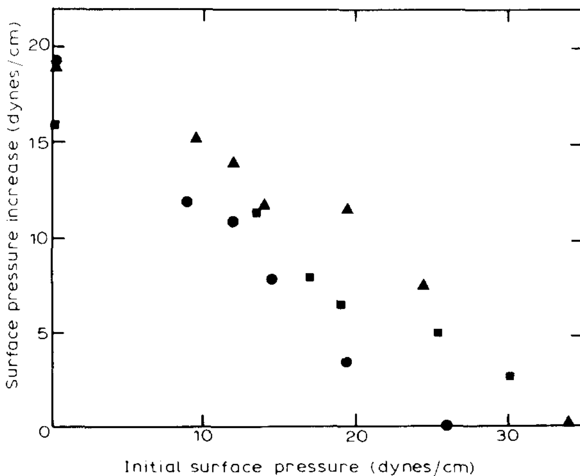

Fig. 1. Effects of native eel acetylcholinesterase on the surface pressure of a monolayer made of total erythrocyte lipids measured at pH 3.0, 4.5 and 7.0. Surface pressure increases are represented as a function of the initial surface pressure. The subphases contained 2 mM $CaCl_2$. They were buffered with 10 mM sodium acetate, pH 3.0 (▲); pH 4.5 (■) and Tris · HCl, pH 7.0 (●).

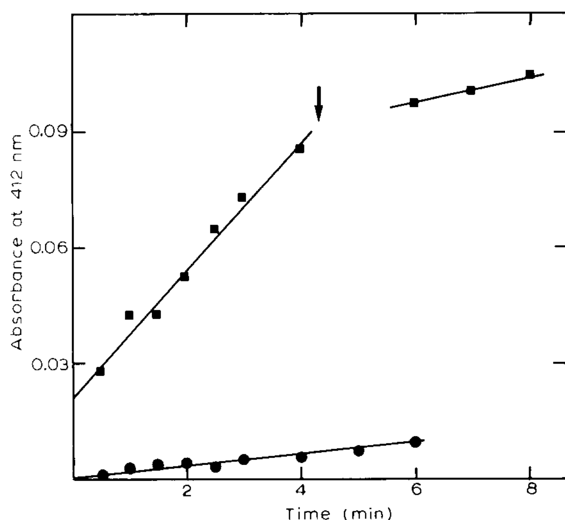

Fig. 2. Measurement of the eel acetylcholinesterase activity present in a lipid film. Acetylcholinesterase (140 I.U., 20 μg in 40 μl of 0.01 M sodium phosphate (pH 7.4)/1 M NaCl/0.05% (w/v) NaN_3) was injected underneath a monolayer made of total erythrocyte lipids. The subphase was 0.01 M Tris · HCl (pH 7.0)/2 mM $CaCl_2$. Initial surface pressure was 18.0 dynes/cm. After 1 h it was 21.8 dynes/cm. The mixed film was washed by five changes of the subphase. The enzyme activity was determined after addition of acetylthiocholine (final concentration 1 mM) by measuring the thiocholine release into the subphase: During the first 4 min, samples (100 μl) were withdrawn every 30 s and added to 100 μl of a solution of decamethonium (final concentration, 20 mM). An aliquot (100 μl) of the resulting mixture was added to 3 ml of a solution of Nbs_2 (0.125 mM). The absorbance was measured at 412 nm (■). After 4 min the mixed film was removed by aspiration with a Pasteur pipet (↓) and residual enzyme activity was measured in the same compartment. As control spontaneous hydrolysis of acetylthiocholine was measured (●).

pH 3 than at pH 7. With the same enzyme preparation, the penetration rate was about five times higher at pH 3.0 than at pH 4.5 or 7.0. When other lipids (phosphatidylcholine and sphingomyelin from human red cell membranes or total lipids extracted from the electric organ of the eel or O-CH_3-phosphatidylcholine) were used the surface pressure increases and penetration rates were similar to those described above.

Native eel acetylcholinesterase occurs in multiple molecular forms [29]. These were modified by treatment with trypsin-Sepharose and thereafter used for penetration studies. With a monolayer made of erythrocyte lipids the modified enzyme yielded at pH 7.4 a surface pressure increase of 3.9 dynes/cm and a penetration rate of 3.1 dynes/cm per 10 min at an initial surface pressure of 18.6 dynes/cm.

Two additional methods were used to show the presence of acetylcholinesterase in erythrocyte lipid films:

(a) Acetylcholinesterase activity was measured by adding acetylthiocholine and determining thiocholine released into the subphase. After the unpenetrated enzyme was removed by repeated washes (Fig. 2) a total activity of 0.45 unit was found under these conditions, which represented 0.32% of enzyme activity originally injected. When the mixed film was removed by aspiration, the thiocholine release into the subphase dropped to the blank value. Table I shows the

TABLE I

ACETYLCHOLINESTERASE ACTIVITY IN MIXED FILMS

Using erythrocyte acetylcholinesterase, an increase in surface pressure of 7.4 dynes/cm and a penetration rate of 1.2 dynes/cm per 10 min at an initial surface pressure of 13.4 dynes/cm were obtained.

pH	Initial surface pressure (dynes/cm per 10 min)	Increase in surface pressure (dynes/cm)	Enzyme activity (I.U.)
3.5	13.9	13.2	0.266
6.0	17.3	6.2	0.140
7.0	10.2	6.5	0.147
7.0	14.8	6.2	0.091
7.0	18.0	3.8	0.455
7.0	18.4	4.6	0.196
7.0	20.2	4.6	0.112
7.0	20.5	3.5	0.343
7.0	21.0	3.5	0.063

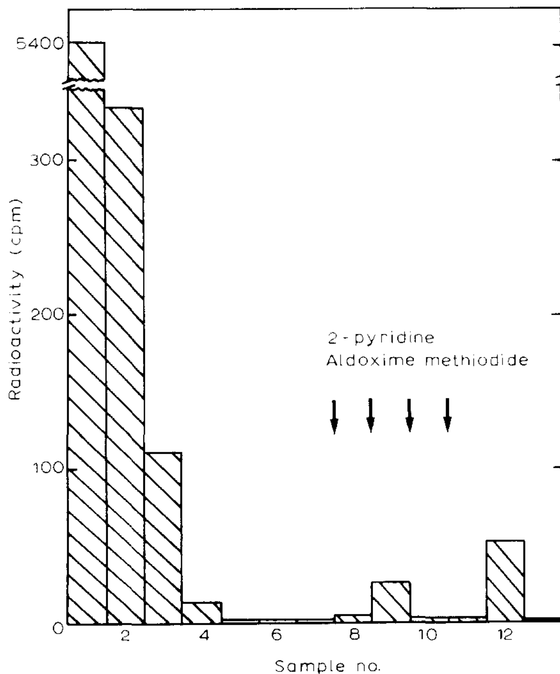

Fig. 3. Effect of 2-pyridine-aldoxime methiodide on [^{3}H]iPr$_2$*P*-acetylcholinesterase in a lipid film. [^{3}H]-iPr$_2$*P*-acetylcholinesterase (20 μg in 50 μl 0.01 M sodium phosphate (pH 7.4)/1 M NaCl, 0.05% (w/v) NaN_3) was injected underneath a monolayer made of total erythrocyte lipids. The subphase was 0.01 M Tris · HCl (pH 7.0)/2 mM $CaCl_2$. After 1 h the mixed film was washed six times with fresh subphase at 10-min intervals. Then 2-pyridine-aldoxime methiodide at a final concentration of 1 mM was injected (↓) and after 10 min the film was moved onto the other subphase. 2-Pyridine-aldoxime methiodide was injected as before and the entire procedure repeated two more times. Finally the film together with the subphase were removed by aspiration and the empty compartment washed with 7 ml of subphase buffer. All the samples were lyophilized and the residues were taken up in 200 μl water. Methanol (6 ml) and BBOT (10 ml of a solution of 4 g/l toluene) were added and the samples were counted for radioactivity three times for 10 min. Background values of 16 cpm were subtracted from experimental values. Samples 1—7 were subphases from the washing procedure; samples 8—11, subphases obtained after injection of 2-pyridine-aldoxime methiodide; sample 12, subphase containing the lipid film and sample 13, subphase containing the control buffer.

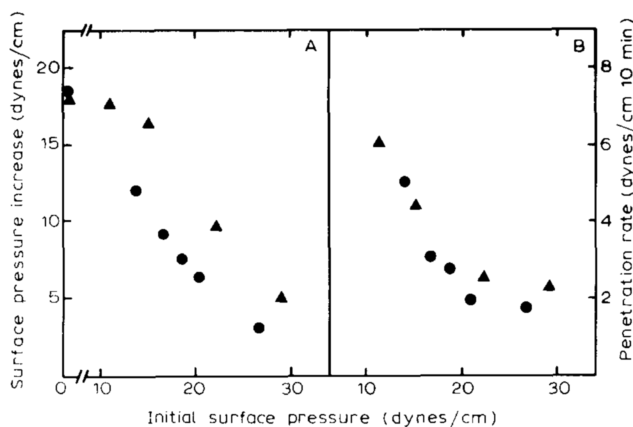

Fig. 4. Effect of *Torpedo* acetylcholine receptor on the surface pressure of a monolayer made of total erythrocyte lipids measured at pH 3.0 and 7.0. Surface pressure increases (A) and penetration rates (B) are represented as a function of the initial surface pressure. The subphases contained 2 mM $CaCl_2$ and were buffered with 0.01 M sodium acetate (pH 3.0) (▲) and 0.01 M Tris · HCl (pH 7.0) (●).

increase in surface pressure and the enzyme activity after penetration of acetylcholinesterase into lipid monolayers of increasing initial surface pressures.

(b) Acetylcholinesterase labelled with [^{3}H]iPr$_2$*P*-F was injected underneath an erythrocyte lipid monolayer (Fig. 3). After penetration the excess enzyme was removed by repeated washes. When 2-pyridine-aldoxime methiodide was added to the subphase, radioactive material was released from the film. Radioactive material was also found in the lipid film when it was finally removed by aspiration.

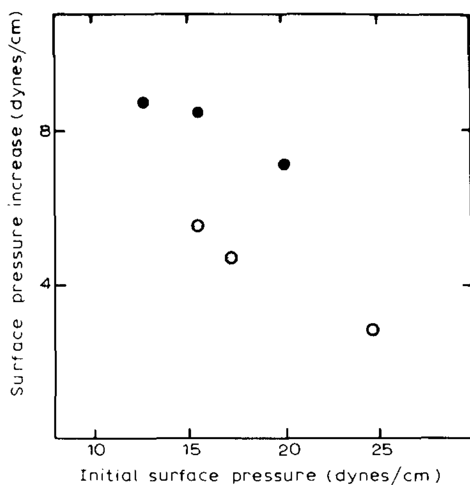

Fig. 5. Effect of *Torpedo* acetylcholine receptor on the surface pressure of a monolayer made of total erythrocyte lipids measured in presence (2 mM, ●) and absence of Ca^{2+} (○). Surface pressure increases are represented as a function of the initial surface pressure. The subphases were buffered with 0.01 M Tris · HCl (pH 7.0).

Penetration of acetylcholine receptor into lipid monolayers

Surface pressure increases were measured at different initial surface pressures and two pH values when acetylcholine receptor was injected into the subphase of lipid monolayers made of erythrocyte lipids (Fig. 4A). The total increases in the surface pressure were higher at pH 3.0 than at 7.0. Fig. 4B relates the penetration rate to the initial surface pressure at these two pH values. When these measurements were performed in absence of calcium the surface pressure increases obtained were lower than in its presence (Fig. 5). The penetration rates were also about two times lower under these conditions.

The presence in the lipid film of the receptor was checked by one additional method. A complex made of α-[^{3}H]toxin and acetylcholine receptor was allowed to penetrate into an erythrocyte lipid monolayer. After the unpenetrated complex was removed by repeated washes, hexamethonium (final concentration, 0.1 M) was injected underneath the film (Fig. 6). Under these conditions about 44% of the radioactive material present in the lipid film was released into the subphase. Of the amount of complex injected into the subphase about 3.5% was actually incorporated.

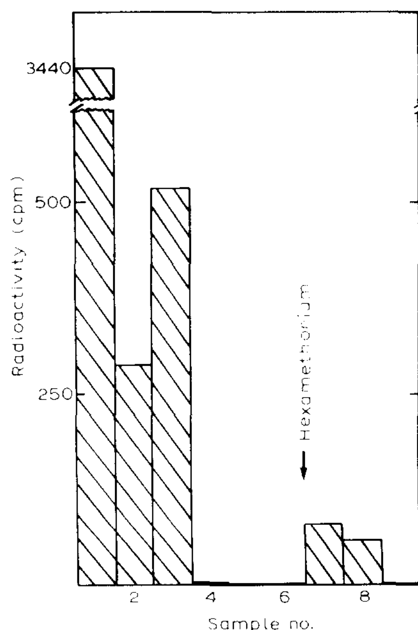

Fig. 6. Effect of hexamethonium on the α-[^{3}H]toxin-acetylcholine receptor complex in a lipid film. α-[^{3}H]Toxin-acetylcholine receptor (20 μg in 200 μl) was injected underneath a monolayer made of total erythrocyte lipids. The subphase was 0.01 M Tris · HCl (pH 7.0)/2 mM $CaCl_2$. After 1 h, the mixed film was washed five times with a fresh subphase at 10-min intervals. Then hexamethonium at a final concentration of 0.1 M was injected (↓). After 10 min the mixed film was moved onto the other subphase and aspirated with a Pasteur pipet. All the samples were collected, lyophilized and counted for radioactivity as described in Fig. 3. Samples 1—6 were suphases from the washing procedure; sample 7, subphase obtained after injection of hexamethonium; sample 8 subphase containing the lipid film and sample 9, subphase containing 7 ml of the buffer used to wash the compartment after removing sample 8.

Discussion

The monolayer technique has been used by several investigators to study lipid-protein interactions [28,30—35]. The penetration of a protein into a lipid film may be characterized by measuring the increase in surface pressure and the penetration rate. The hydrophobicity of the protein, the charge of the protein and the lipid and the ionic strength of the subphase influence these two factors.

Interaction of acetylcholinesterase with lipid monolayers

Depending on the source, acetylcholinesterase can be regarded as peripheral or integral membrane protein according to the distinction made by Singer and Nicolson [36]. The eel enzyme, readily solubilized by high salt solutions, belong to the former class [37] whereas the one from human erythrocytes, solubilized only in presence of detergents to the latter [20].

In this context the erythrocyte enzyme was expected to be readily incorporated into a lipid monolayer especially when the lipids used for the monolayer were extracted from the same membranes as the enzyme. As a matter of fact penetration indeed occurred as shown by the recorded pressure increase of 7.4 dynes/cm at an initial surface pressure of 13.4 dynes/cm. Contrary to our expectation, however, this value was only slightly higher than those obtained with eel acetylcholinesterase. Detergent-depleted erythrocyte acetylcholinesterase assumes aggregated forms of high molecular weights. The hydrophobic sites, which in the native membrane interact with the lipid bilayer, thus become masked. This could explain the fact that the red cell enzyme does not give the increases in surface pressure expected for an integral membrane protein. According to these first findings we decided to mainly use the more readily available eel enzyme for further studies. It showed a surface activity of about 18 dynes/cm at pH 7 in absence of lipids. In their presence substantial increases in pressure were observed for initial surface pressures ranging from 10 to 24 dynes/cm. This indicates that penetration had occurred. When the initial surface pressure was superior to 24 dynes/cm, no further increase was recorded. No substantial differences were observed when monolayers were made from total eel lipids or total erythrocyte lipids. After this finding the well defined lipids from red cell membranes were used for further studies.

Increases in surface pressures were recorded at pH values of the subphase of 3.0, 4.5 and 7.0. Higher increases were obtained by lowering the pH. According to Quinn and Dawson [30,31] such findings could indicate an increase in hydrophobic area of the protein due to conformational changes obtained at low pH values. Penetration rates were also recorded at pH values of the subphase of 3.0, 4.5 and 7.0. At pH 3.0 a 5-fold higher rate was obtained than at the two other pH values tested, which could reflect more favorable electrostatic interactions at pH 3.0, thus enhancing lipid protein complex formation [33]. This is of special interest with eel acetylcholinesterase since its isoelectric point is 3.2 [38]. However, the contribution of the electrostatic interaction must be minor as penetration still occurs when a homogeneous film of positively charged lipid (O-CH_3-phosphatidylcholine) is used even at pH 3, a value at which the enzyme is also positively charged.

Native eel acetylcholinesterase assumes highly asymmetric forms which in

electron microscopy appear as grape-like structures composed of a tail with clusters of subunits attached to it. Trypsin treatment of these forms yields a more globular enzyme devoid of the tail-like extension [39]. In situ this extension might be responsible for the attachment of the enzyme to the membrane [40,41]. The values obtained for increases in surface pressures and penetration rates at pH 7 indicate that in absence of the tail-like structure, the modified enzyme penetrates the film as well as the unmodified one. We therefore conclude that at least in our system the tail is not an essential molecular feature for lipid binding. We rather suggest that mainly hydrophobic interactions between the globular part of the enzyme and the lipids are responsible for the association with the monolayer. This is compatible with the idea that the enzyme spreads on the surface of the lipids and the tails form a reticulum-like structure [42].

The presence of the enzyme in the lipid film was confirmed by two different experiments. Their results indicate that the active site of the enzyme is oriented towards the aqueous phase as it occurs in situ. It should, however, be noticed that only a small percentage of the enzyme injected into the subphase actually penetrated the film.

Interaction of acetylcholine receptor with lipid monolayers

Another protein component of cholinergically excitable membranes to be tested in this system is the acetylcholine receptor, which is regarded as an integral membrane protein [43]. It can be readily obtained in a pure form from the electric organ of the fish *T. marmorata* [11]. Prior to penetration studies excess of the detergent used to solubilize the receptor was removed. In order, however, to keep the protein in solution a minimal amount of 100 μg/ml of Triton X-100 is necessary which itself acts as a surface active compound. At the final concentration of 0.45 μg/ml in the Langmuir trough the detergent was no longer responsible for the observed changes in surface pressure. It could, however, mask the hydrophobic regions of the protein. As a consequence the increases in surface pressures and penetration rates will not necessarily reflect the hydrophobic interaction of the protein with its native environment. These facts should be kept in mind while analyzing the present results obtained with the acetylcholine receptor at two pH values. These experiments confirm that the presence of calcium is necessary for optimal interactions of acetylcholine receptor with lipids [2].

The presence of the acetylcholine receptor in the lipid film was confirmed by an experiment in which tritiated α-toxin-acetylcholine receptor complex was used from which the labelled toxin could selectively be displaced after penetration. This result shows also that the active site of the receptor was available to the competing agent. Though again only a small percentage of the molecules injected into the subphase actually penetrated the film, the amount nevertheless was larger than that obtained with acetylcholinesterase. The present result show that both proteins can indeed by incorporated into lipid monolayers. However, the method does not allow to exactly describe the events occurring upon protein binding to the lipids. We cannot exclude that conformational changes of the proteins at the air-water interface are at least partly responsible for the observed increases in surface pressure. Thus the

environment for the proteins in the lipid monolayer could be quite different from the one in the native membrane.

Acknowledgements

This work was supported by a grant No. 134 of the Fritz Hoffmann-La Roche Stiftung zur Förderung wissenschaftlicher Arbeitsgemeinschaften in der Schweiz and a grant No. 3.551.0.75 of the Swiss National Science Foundation.

References

1 Shamoo, A.E. and Eldefrawi, M.E. (1975) J. Membrane Biol. 25, 47—63
2 Eldefrawi, M.E., Eldefrawi, A.T. and Shamoo, A.E. (1975) Ann. N.Y. Acad. Sci. 264, 183—202
3 Bradley, R.J., Howell, J.H., Romine, W.O., Carl, G.F. and Kemp, G.E. (1976) Biochem. Biophys. Res. Commun. 68, 577—584
4 Romine, W.O., Goodall, M.C., Peterson, J. and Bradley, R.J. (1974) Biochim. Biophys. Acta 367, 316—325
5 Goodall, M.C., Bradley, R.J., Saccomani, G. and Romine, W.O. (1974) Nature 250, 68—70
6 Michaelson, D.M. and Raftery, M.A. (1974) Proc. Natl. Acad. Sci. U.S. 71, 4768—4772
7 Leuzinger, W. and Schneider, M. (1972) Experientia 28, 256—257
8 Jain, M.K., Mehl, L.E. and Cordes, E.H. (1973) Biochem. Biophys. Res. Commun. 51, 192—197
9 Jain, M.K. (1974) Arch. Biochem. Biophys. 164, 20—29
10 Montal, M. and Mueller, P. (1972) Proc. Natl. Acad. Sci. U.S. 69, 3561—3566
11 Klett, R.P., Fulpius, B.W., Cooper, D., Smith, M., Reich, E. and Possani, L.D. (1973) J. Biol. Chem. 248, 6841—6853
12 Eldefrawi, M.E. and Eldefrawi, A.T. (1973) Arch. Biochem. Biophys. 159, 362—373
13 Meunier, J.-C., Sealock, R., Olsen, R. and Changeux, J.-P. (1974) Eur. J. Biochem. 45, 371—394
14 Raftery, M.A., Bode, J., Vandlen, R., Michaelson, D., Deutsch, J., Moody, T., Ross, M.J. and Stroud, R.M. (1975) in Protein-Ligand Interactions (Sund, H. and Blauer, G., eds.), pp. 328—355, Walter de Gruyter, Berlin
15 Berman, J.D. and Young, M. (1971) Proc. Natl. Acad. Sci. U.S. 68, 395—398
16 Hopff, W.H., Riggio, G. and Waser, P.G. (1973) FEBS Lett. 35, 220—222
17 Dudai, Y., Silman, I., Shinitzky, M. and Blumberg, S. (1972) Proc. Natl. Acad. Sci. U.S. 69, 2400—2403
18 Wiedmer, T., Brodbeck, U., Zahler, P. and Fulpius, B. (1976) Abstr. Commun. 10th Int. Congr. Biochem. Abstr. 05-1-308
19 Wiedmer, T., Gentinetta, R. and Brodbeck, U. (1974) FEBS Lett. 47, 260—263
20 Ott, P., Jenny, B. and Brodbeck, U. (1975) Eur. J. Biochem. 57, 469—480
21 Ellman, G.L., Courtney, D.K., Andres, V. and Featherstone, R.M. (1961) Biochem. Pharmacol. 7, 88—95
22 Cooper, D. and Reich, E. (1972) J. Biol. Chem. 247, 3008—3013
23 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
24 Parikh, I., March, S. and Cuatrecasas, P. (1974) in Methods in Enzymology (Jakoby, W.B. and Wilcheck, M., eds.), Vol. XXXIV, pp. 77—102, Academic Press, New York
25 Dodge, J.T., Mitchell, C. and Hanahan, D.J. (1963) Arch. Biochem. Biophys. 100, 119—130
26 Folch, J., Lees, M. and Sloan-Stanley, G.H. (1957) J. Biol. Chem. 226, 497—509
27 Kramer, R., Schlatter, Ch. and Zahler, P. (1972) Biochim. Biophys. Acta 282, 146—156
28 Morse, II, P.D. (1974) J. Supramol. Struct. 2, 60—70
29 Wermuth, B., Ott, P., Gentinetta, R. and Brodbeck, U. (1975) in Symposium on Cholinergic Mechanisms (Waser, P.G., ed.), pp. 299—308, Raven Press, New York
30 Quinn, P.J. and Dawson, R.M.C. (1969) Biochem. J. 113, 791—803
31 Quinn, P.J. and Dawson, R.M.C. (1969) Biochem. J. 115, 65—75
32 Quinn, P.J. and Dawson, R.M.C. (1970) Biochem. J. 116, 671—680
33 Morse, II, P.D. and Deamer, D.W. (1973) Biochim. Biophys. Acta 298, 769—782
34 Demel, R.A., London, Y., Geurts van Kessel, W.S.M., Vossenberg, F.G.A. and van Deenen, L.L.M. (1973) Biochim. Biophys. Acta 311, 507—519
35 London, Y., Demel, R.A., Geurts van Kessel, W.S.M., Zahler, P. and van Deenen, L.L.M. (1974) Biochim. Biophys. Acta 332, 69—84
36 Singer, S.J. and Nicolson, G.L. (1972) Science 175, 720—731

37 Rosenberry, T.L. (1976) in The Enzymes of Biological Membranes (Martonosi, A., ed.), Vol. 4, pp. 331—363, John Wiley and Sons, London
38 Brodbeck, U., Ott, P. and Wiedmer, T. (1975) Croat. Chem. Acta 47, 201—210
39 Rieger, F., Bon, S., Massoulié, J. and Cartaud, J. (1973) Eur. J. Biochem. 34, 539—547
40 Massoulié, J. and Rieger, F. (1969) Eur. J. Biochem. 11, 441—455
41 Dudai, Y. and Silman, I. (1973) FEBS Lett. 30, 49—52
42 Silman, I. and Dudai, Y. (1973) in Protides of the Biological Fluids (Peeters, H., ed.), pp. 257—261, Pergamon Press, Oxford
43 Fulpius, B.W. (1976) in Motor Innervation of Muscle (Thesleff, S., ed.), pp. 1—29, Academic Press, New York