PRESENCE OF TWO FORMS OF ACETYLCHOLINE RECEPTOR WITH DIFFERENT ISOELECTRIC POINTS IN THE ELECTRIC ORGAN OF *ELECTROPHORUS ELECTRICUS* AND THEIR CATALYTIC INTERCONVERSION *IN VITRO*

Vivian Itzhak TEICHBERG and Jean-Pierre CHANGEUX
Neurobiologie Moléculaire - Institut Pasteur, 25 rue du Dr. Roux 75015 Paris, France

Received 22 June 1976

1. Introduction

The acetylcholine receptor (AChR) is not uniformly distributed on the surface of the striated muscle fiber or of the electroplaque and its density under the nerve terminals may exceed by a factor of 100 or even 1000 that present in extrasynaptic areas [1]. Physiological [2,3], pharmacological [4] and biochemical [5,6] differences have been reported between these two classes of receptor but the structural basis of these differences yet remains to be understood. For instance, do separate genes code for the extra and subsynaptic receptors? Or, alternatively, would the different forms of the AChR result from a covalent modification of a single protein species as commonly found with regulatory [7] or membrane bound [8] proteins?

To approach this problem, we have used the electric organ of *Electrophorus electricus* for which autoradiographic data indicate that the AChR is present in both subsynaptic and extrasynaptic areas in almost equal amounts [9]. In this communication, we present evidence that in crude detergent extracts of membrane fragments from *E. electricus* electric tissue, AChR is present under two forms with different isoelectric points. Moreover a catalytic interconversion of each form into the other has been achieved in vitro suggesting that a chemical modification may be responsible for the observed difference.

The implications of these results are discussed in terms of a theory of selective stabilization of developing synapses [10,21].

2. Materials and methods

2.1. *Extraction of the cholinergic receptor*

40 g of frozen or fresh tissue of electric organ were homogenized into 80 ml of PNM (10^{-4} M PMFS, a protease inhibitor, 0.02% NaN_3, 0.014 M 2-mercaptoethanol in H_2O, pH 6.2) for 2 min at 0°C using a Virtis blender at medium speed. This homogenate was centrifuged for 30 min, 20 000 *g* at 4°C; the pellet was resuspended in 20 ml PNM, and a 20% solution of Triton X-100 was added to obtain a final Triton X-100 concentration of 5%. The extraction was carried out under gentle stirring at room temperature for 60 min. The suspension was then centrifuged for 60 min, 4°C at 100 000 *g* and the supernatant (S_2) collected and kept at 4°C.

2.2. *Isoelectric focusing*

The isoelectric focusing was carried out at 4°C for 12 h in a 80 ml electrofocusing glass column [11], along a 52 ml sucrose gradient (44–16%) containing 1% pH 4–6 Ampholyte, 0.1% Triton, 0.4 M urea in PNM.

3. Results

Fig.1 shows the results of an experiment of isoelectric focusing performed on a crude Triton X-100 extract of membrane fragments prepared from electric organ of *E. electricus*. After the run, AChR was

North-Holland Publishing Company – Amsterdam

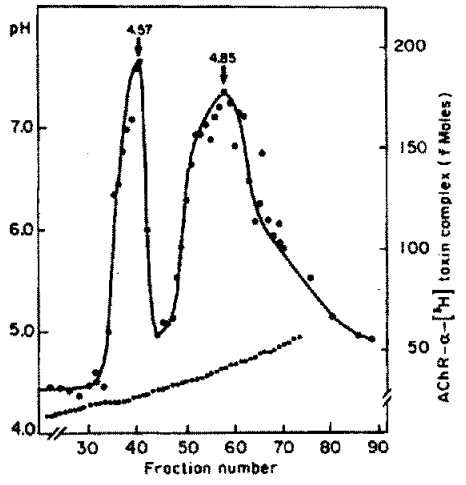

Fig.1. Isoelectric focusing profile of S_2 fraction. 118 pM of $\alpha[^3H]$toxin binding sites were applied. Electrofocusing was carried out at constant power for 12 h. 340 μl fractions were collected. The pH value of each fraction was determined at 4°C. Each fraction was then neutralized with 2 M Tris buffer pH 7.0. AChR was assayed according to Olsen et al. [12]. The specific activity of the $\alpha[^3H]$toxin batch used was 30 Ci/mmol and 61% of the α-toxin molecules were pharmacologically active. The yield in toxin sites recovered was 56%.

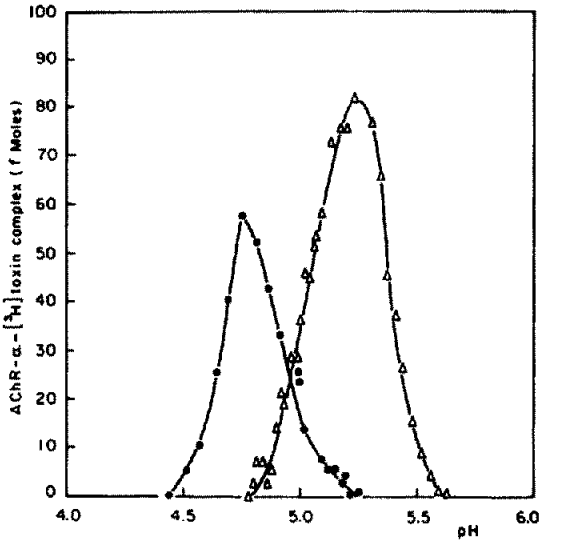

Fig.2. Isoelectric focusing profiles of AChR forms. Acidic form of AChR (○ – ○) 13 pmol of $\alpha[^3H]$toxin binding sites. Alkaline form of AChR (△ – △) 30 pmol of $\alpha[^3H]$toxin binding sites analysed under conditions as described fig.1.

assayed in the collected 340 μl fractions with $\alpha[^3H]$ toxin (30 Ci/mmol) from *N. nigricollis* [12]. The profile of $\alpha[^3H]$toxin binding sites consists of two peaks: one focusing at pH 4.57, the other at pH 4.85.

It could be shown that the pH values at which AChR focuses did not depend on the place of application of the sample in the column and that they could vary from run to run by up to 0.12 pH units. In any event, two peaks of AChR were always observed and separated by at least 0.22 pH units. In order to challenge the possibility that the two forms of AChR may result from a reversible equilibrium established within the column, the fractions composing each toxin binding peak were pooled, dialyzed against 0.1% Triton X-100 in PNM buffer concentrated on DEAE cellulose [13] and resubmitted to isoelectric focusing. Fig.2 shows that each toxin binding peak refocuses as a single species indicating that the two peaks observed in fig.1 represent two distinct and stable forms of AChR. Although each form keeps its relative acidity with respect to the other, they do not refocus at exactly the same isoelectric pH as the fractions from which they originate. The reasons for this variability are not known.

Brockes and Hall [5] have reported differences in the binding properties of two AChR forms isolated from denervated rat diaphragm. We therefore measured, at 20°C, the rate constant of $\alpha[^3H]$toxin binding to each AChR form from *Electrophorus* [14] and found it to be the same in both cases: ($k = 5 \times 10^7$ M^{-1} min^{-1}). The inhibition by d-tubocurarine of the initial rate of $\alpha[^3H]$toxin binding to each AChR form gave an identical protection constant of 2×10^{-7} M. This latter finding is in complete agreement with the work of Alper et al. [15].

The two distinct forms of AChR may either reflect differences in primary structure or result from a covalent modification of a single molecular species. To test this second alternative attempts were made to obtain an interconversion in vitro between the two forms. First the possibility of an heterogeneity of AChR forms due to the reduction of a disulfide bridge in the receptor by 2-mercaptoethanol was eliminated since the two forms of AChR could still be observed in the absence of reducing agent. An aliquot of the crude extract S_2 was then incubated

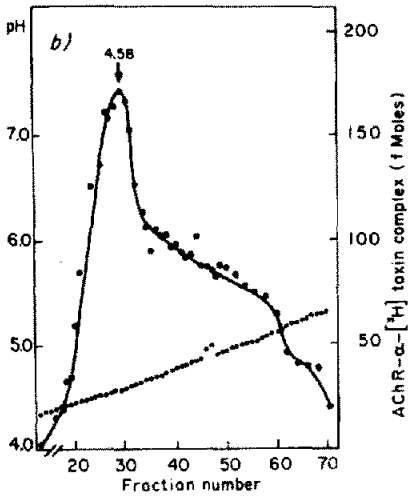

Fig.3. Isoelectric focusing profile of S_2 fraction after 0.1 M NaF treatment at 37°C for 30 min and dialysis at 4°C against large volumes of PNM containing 0.1% Triton X-100. 168 pmol of α[^{3}H]toxin binding sites were applied. The yield in toxin binding sites after incubation at 37°C and dialysis was 76% and the recovery in AChR after isoelectric focusing 54%.

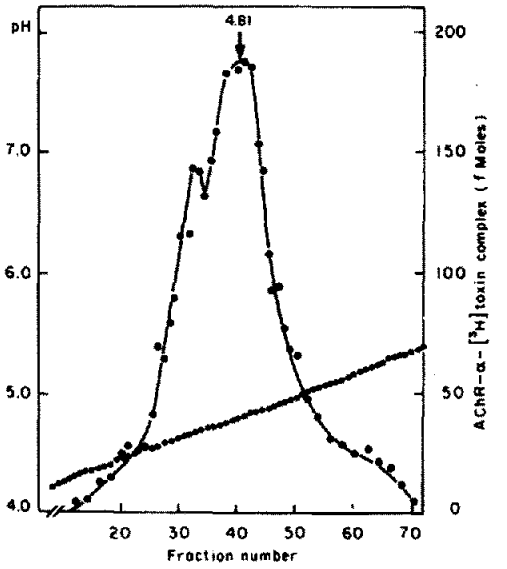

Fig.4. Isoelectric focusing profile of S_2 fraction after 0.1 M NaCl treatment at 37°C for 30 min and dialysis at 4°C against large volumes of PNM containing 0.1% Triton X-100. 198 pmol of α[^{3}H]toxin binding sites were applied. The yield in toxin binding sites after incubation at 37°C and dialysis was 76% and the recovery in AChR after the isoelectric focusing 49%.

for 30 min at 37°C in the presence of 0.1 M NaF in PNM. The extract was dialysed for 24 h against 0.1% Triton X-100 in PNM and submitted to isoelectric focusing; fig.3 shows the resulting profile of toxin binding activity. When the same experiment was carried out in the presence of 0.1 M NaCl instead of NaF, then, an entirely different profile was obtained (fig.4).

Clearly, after treatment at 37°C only one of the two forms of the AChR remains: the acidic one with NaF, the more alkaline one with NaCl. Treatment at 37°C for 30 min without any salt did not modify the profile shown in fig.1.

Since a significant inactivation of AChR takes place during the heat treatment and the electrofocusing, two alternative interpretations may account for the observed results. Either one of the two forms is selectively inactivated or an interconversion between the two forms takes place. First of all, it could be shown that the electrofocusing in itself did not cause a selective inactivation of one of the two forms. But this may have taken place during the heat treatment. The following experiment rules out this possibility.

The crude extract, S_2, was first incubated in the presence of 0.1 M NaF under conditions which favor the acidic form. Then, the extract was dialysed and heated again at 37°C but in the presence of 0.1 M NaCl. The profile obtained after this second treatment is, again, identical to that shown in fig.4. The alkaline peak which disappeared after the first treatment in NaF, reappeared after the second exposure to NaCl.

These results are taken as strong indication that the treatment of a crude extract of AChR at 37°C in the presence of NaCl or NaF leads to an interconversion between acidic and alkaline forms rather than to a selective inactivation of anyone of these forms. It should be noticed however that in treating the crude membrane extract with NaF, the conversion of the alkaline peak into the acidic one was not always complete and a peak was often observed around pH 4.85. This was not the case upon treatment of the crude membrane extract with NaCl which resulted always in a complete conversion into the AChR alkaline form.

4. Discussion

In rat diaphragm muscle before and after denervation, Brockes & Hall [5] have found two forms of α[^{125}I]bungarotoxin–AChR complexes; In crude Triton X-100 extracts of membrane fragments from *E. electricus* electric organ, the cholinergic receptor protein is also present under at least two discrete forms with different isoelectric points. Since the difference in isoelectric point between the purified AChR (pH 4.7) from *E. electricus* [16] and its toxin complex (pH 5.15) [17] is about 0.45 pH units, the isoelectric properties of the two AChR forms from *Electrophorus* are very similar if not identical to those of the subsynaptic and extrasynaptic receptors from rat diaphragm. By high resolution autoradiography of the α[^{3}H]toxin-labelled electroplaque from *E. electricus*, Bourgeois et al. [9] have shown that almost equal amounts of subsynaptic and extrasynaptic AChR are present in this cell. Interestingly, this proportion is close to that found between the two forms with different isoelectric points observed after detergent dissolution. It is therefore quite plausible that these two forms correspond also to extrasynaptic and subsynaptic AChR.

Whatever the origin of these two forms is, it appears clear that an in vitro treatment in the presence of salts modifies their isoelectric point. Despite the fact that these two stable forms of AChR are not yet identified chemically but distinguished only on the basis of their isoelectric behavior, the most likely interpretation of the data is that an interconversion between the two forms takes place in vitro. One possible candidate for the modification is a phosphorylation–dephosphorylation reaction catalysed by protein kinases and phosphoprotein phosphatases present in the crude extract. The known inhibition of phosphoprotein phosphatases by NaF [18] would explain the differential effect of NaCl and NaF. In the presence of NaF and *provided* that the cofactors needed by the protein kinase are present in the crude membrane extract, AChR would be phosphorylated and therefore should focus at a more acid pH whereas in the presence of NaCl the phosphorylated AChR should be dephosphorylated by the phosphoprotein phosphatase and focus at a more basic pH. The reproductibility of the conversion of the acidic AChR into the alkaline species in contradistinction to the conversion observed in the presence of NaF could be explained by the stringent requirement by the protein kinase of cofactors that may not be present or active in each of our preparations.

The functional significance of these two forms is still hypothetical. Among others, two interpretations appear plausible: (1) Following Greengard and co-workers [8], the two forms might be involved in the regulation of the permeability of the postsynaptic membrane by the neurotransmitter. (2) Alternatively, the covalent modification may be at the origin of striking differences in *stability* noticed between the subsynaptic and extrasynaptic receptor [19,20]. This hypothesis has been recently applied to the development of the neuromuscular junction [10,21] and may, in particular, account for the selective accumulation of receptor under the nerve terminal or 'localisation' process. In the developing myotubes, the AChR would be present in its 'unmodified' form, labile and mobile. Under the developing nerve terminal, the postulated covalent modifications would immobilize the protein and render it resistant to degradation. If one further assumes [21] that the synthesis of the labile form of the AChR stops in the postsynaptic cell when the electrical activity of the myotube starts, then, the localisation process could be viewed as the management of a finite stock of receptor protein via lateral diffusion, covalent modification and selective degradation. As a consequence, the density of the receptor would rapidly increase under the nerve terminal and decrease in extrasynaptic areas.

Acknowledgements

We thank Dr P. Boquet for a gift of α-toxin and Drs Menez, Morgat and Fromageot who tritiated it.

We are grateful to Drs Grünhagen, Popot, Sobel and Weber for helpful discussions.

This work was done during the tenure of a research fellowship of Muscular Dystrophy Association of America to V.I.T.

This work was supported by funds from the Centre National de la Recherche Scientifique, the Délégation Générale à la Recherche Scientifique et Technique, the Collège de France, the Commissariat à l'Energie Atomique and the National Institutes of Health.

References

[1] Hartzell, H. C. and Fambrough, D. M. (1972) J. Gen. Physiol. 60, 248–262.
[2] Miledi, R. (1960) J. Physiol. 151, 1–23.
[3] Beranek, R. and Vyskosil, F. (1967) J. Physiol. 188, 53–66.
[4] Chiu, T. H., Lapa, A. J., Barnard, E. A. and Albuquerque, E. X. (1974) Exp. Neurol. 43, 399.
[5] Brockes, J. P. and Hall, Z. W. (1975) Biochemistry 14, 2092–2099, and 14, 2100–2106.
[6] Berg, D. K. and Hall, Z. W. (1975) J. Physiol. 252, 771–789.
[7] Fischer, E. H., Heilmeyer, L. M. G. Jr. and Haschke, R. H. (1970) in: Current Topics in Cellular Regulation, Vol. 4.
[8] Greengard, P. (1976) Nature 260, 101–108.
[9] Bourgeois, J. P., Popot, J. L., Ryter, A. and Changeux, J. P. (1973) Brain Res. 62, 557–563.
[10] Changeux, J. P. (1974) in: Cell Surface in Development. (A. A. Moscona, ed.) 207–220, John Wiley and Sons, Inc.
[11] Joseleau-Petit, D. and Kepes, A. (1975) Biochim. Biophys. Acta 406, 36–49.
[12] Olsen, R., Meunier, J. C. and Changeux, J. P. (1972) FEBS Lett. 28, 96–100.
[13] Meunier, J. C., Scalock, R., Olsen, R. and Changeux, J. P. (1974) Eur. J. Biochem. 45, 371–394.
[14] Weber, M. and Changeux, J. P. (1974) Mol. Pharmacol. 10, 1–4.
[15] Alper, R., Lowy, J. and Schmidt, J. (1974) FEBS Lett. 48, 130–134.
[16] Biesecker, G. (1973) Biochemistry 12, 4403–4409.
[17] Raftery, M. A., Schmidt, J., Clark, D. G. and Wolcott, R. G. (1971) Biochem. Biophys. Res. Commun. 45, 1622–1629.
[18] Revel, H. R. (1963) Methods in Enzymology, vol. VI, p. 211–214.
[19] Devreotes, P. N. and Fambrough, D. M. (1975) J. Cell Biol. 65, 335–358.
[20] Chang, C. C. and Huang, M. C. (1975) Nature 253, 643–644.
[21] Changeux, J. P. and Danchin, A. (1976) Nature, in the press.