ATP-BINDING PROTEINS IN ACETYLCHOLINE RECEPTOR-ENRICHED MEMBRANES

Adrienne S. GORDON, Richard J. GUILLORY[+], Ivan DIAMOND* and Ferdinand HUCHO[†]

Department of Neurology and the Liver Center, University of California, San Francisco, CA 94143,
[+]*Department of Biochemistry and Biophysics, John A. Burns School of Medicine, University of Hawaii, Honolulu, HI 96822,*
**Department of Neurology and Pediatrics and the Liver Center, University of California, San Francisco, CA 94143, USA* and
[†]*Fachbereich Biologie der Universität, D-7750 Konstanz, FRG*

Received 21 September 1979

1. Introduction

The acetylcholine receptor (AChR) has been shown to be phosphorylated in situ in receptor-enriched membranes from the electric organ of *Torpedo californica* [1] and in a crude detergent extract from *Electrophorus electricus* [2]. Further, AChR-enriched membranes from *Torpedo californica* contain protein kinase [3] and phosphoprotein phosphatase [4] activity and evidence has been presented that these enzymes are probably present in detergent-solubilized crude membrane fractions from *Electrophorus electricus* [5]. However, kinase and phosphatase activities have not been demonstrable in solubilized, purified preparations of AChR. This suggests that the membrane protein kinase and phosphatase are not components of the AChR but are associated with the AChR in receptor-enriched membranes. The purpose of this study, therefore, is to identify polypeptides in receptor-enriched membranes which have ATP binding sites and which might regulate the level of phosphorylation of the AChR.

Arylazido-β-alanyl ATP has been used effectively as a photoaffinity ligand to identify catalytic sites on enzymes for which ATP is a substrate. Here we report the use of arylazido-β-alanyl ATP to identify 3 polypeptides in AChR-enriched membranes which are labeled by the ATP analog and which are not components of the purified AChR. It is possible that 2 of these polypeptides are related to protein kinase and phosphatase activities in AChR-enriched membranes.

2. Materials and methods

AChR-enriched membranes were prepared from *Torpedo californica* as in [6]. The ATP analog, arylazido-β-alanyl ATP was synthesized as in [7] using either unlabeled ATP or [α-^{32}P]ATP; the α-^{32}P-labeled compound had spec. act. 10^9 cpm/μmol. Photoaffinity labeling was performed by irradiating a solution containing AChR-enriched membranes and the photoaffinity label at concentrations indicated in the figure legends. A 10 s irradiation with a 250 W medium pressure mercury lamp (Applied Photophysics Ltd., London) mounted 6.5 cm away from a 0.2 × 1 cm glass cuvette, was sufficient to produce maximum incorporation of the photolabel. Protein kinase activity was determined as in [3]. SDS–polyacrylamide gel electrophoresis was carried out as in [8].

3. Results and discussion

Figure 1 shows that the AChR-enriched membranes used in this study contain ~8 major polypeptides including the 4 subunits of the AChR. After photoirradiation, the ATP photoaffinity analog, arylazido-β-alanyl [α-^{32}P]ATP reacts with only 3 polypeptides in this preparation. The AChR subunits are not labeled. In the absence of irradiation, there is no incorporated radioactivity (data not shown). Thus, comparison of the Coomassie-blue stained gel (B) with the autoradiograph (A) of the same gel in fig.1 shows that the

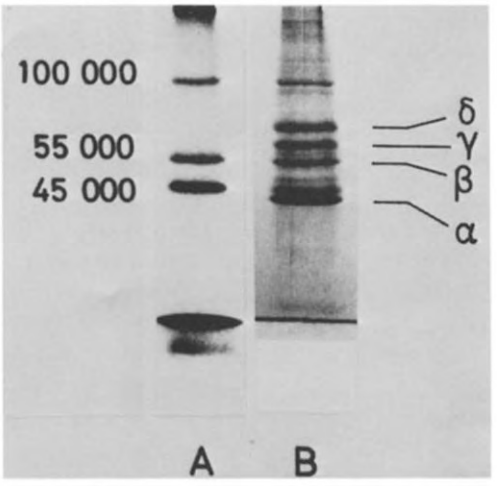

Fig.1. Photoaffinity labeling of AChR-enriched membranes with arylazido-β-alanyl ATP. Autoradiogram (A) and Coomassie blue-stained SDS–polyacrylamide gel (B). Protein (1 mg/ml) was suspended in 15 mM Tris–HCl (pH 7.5) containing 10^{-4} M ouabain and 0.225 mM arylazido-β-alanyl [α-^{32}P]ATP. Irradiation was 4 × 15 s each at room temperature. The samples were chilled in ice following each 15 s irradiation. Total volume was 0.2 ml. SDS electrophoresis was carried out as in [8] in 7.5% acrylamide gels.

affinity ligand-labeled bands migrate with mol. wt 45 000, 55 000 and 100 000 which do not correspond to the molecular weights of the polypeptide components of the purified AChR (α, 42 000; β, 48 000; γ, 62 000; δ, 68 000). The 100 000 mol. wt band appears to be the Na^+,K^+-ATPase which has a similar molecular weight from many different sources [9–11]. This band is not a component of the AChR but is present in variable amounts in receptor-enriched membrane preparations.

Although the function of the labeled polypeptides is unknown, the 45 000 and 55 000 labeled bands could be related to the protein kinase and protein phosphatase activities present in the receptor-enriched membranes. ATP is a substrate for the protein kinase reaction and therefore the enzyme must have an ATP binding site. The phosphatase is inhibited by ATP [4] and therefore may also have a specific binding site for the nucleotide. The specificity of binding of the photoaffinity label for ATP binding sites is shown in fig.2. Unlabeled ATP at increasing concentrations correspondingly inhibited the reaction of the labeled photoaffinity compound. On the other hand, arylazido-β-alanine, i.e., the photolabel without the ATP moiety, did not cause a significant decrease in reactivity of the radioactive affinity label (data not shown). These results strongly suggest that the analog is acting as a photoaffinity probe of adenine nucleotide binding sites in AChR-enriched membranes. If the ATP photoaffinity label reacts irreversibly with a protein kinase in the membranes, then the ATP derivative would be expected to block kinase activity. Figure 3 demonstrates that the photoaffinity label has a striking effect on AChR phosphorylation in receptor-enriched membranes. The membrane-bound AChR was phosphorylated in situ with [γ-^{32}P]ATP as described in the figure legend. Increasing concentrations of unlabeled photoaffinity ligand progressively inhibited receptor phosphorylation. Thus, the ATP photoaffinity label appeared to be reacting with the membrane kinase to inhibit phosphorylation of the membrane-bound AChR. We have also determined that ATP inhibits phosphatase activity in the same membrane preparation. However, because of methodologic limitations we have not been able to study the interaction of the ATP photoaffinity label with

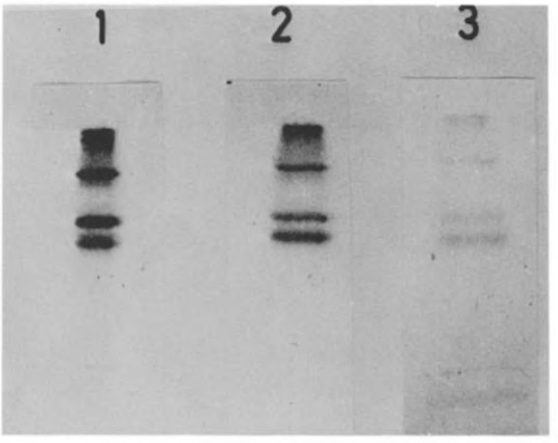

Fig.2. Inhibition of photolabeling with ATP. Autoradiograms of SDS gels. Control as in fig.1 (1). Incubation mixture also contained 50 mM (2) and 100 mM ATP (3).

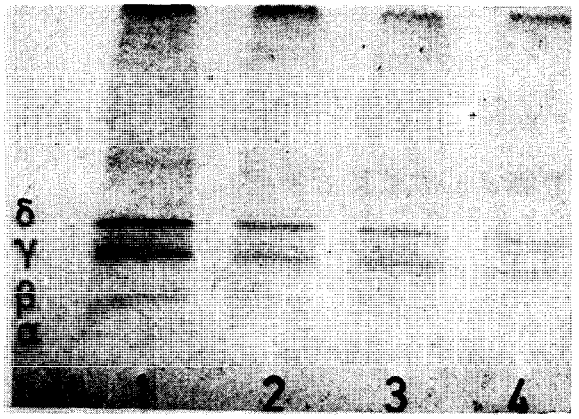

Fig.3. Inhibition of endogenous protein kinase activity in AChR-enriched membranes by arylazido-β-alanyl ATP. Photoaffinity labeling of membranes carried out as in fig.1. Endogenous kinase activity was then determined [1,3]. Membranes containing 50 μg photoaffinity labeled protein were incubated in 0.1 ml for 30 s at 0°C with 5 μM [γ-^{32}P]-ATP (1–2 μCi/tube), 0.25 mM EGTA, 10 mM $MgAc_2$, 0.0625 M Tris–HCl (pH 6.8) and 100 mM KCl. The reaction was stopped by the addition of 15 μl 20% SDS. Electrophoresis was carried out as in [8]. (1) Autoradiogram of a 7.5% SDS–acrylamide gel showing phosphorylation of the γ (mol. wt 62 000) and δ (mol. wt 68 000) bands of the AChR by an endogenous protein kinase. (2) Phosphorylation in the presence of 0.03 mM arylazido-β-alanyl ATP in the dark. (3) Same conditions as before, but with 4 × 15 s irradiation. (4) Phosphorylation after photoaffinity labeling with 0.3 mM arylazido-β-alanyl ATP.

this endogenous membrane phosphoprotein phosphatase.

These results indicate that polypeptides which have ATP binding sites in AChR-enriched membranes are not components of the AChR. A 43 000 mol. wt polypeptide associated with the AChR has been isolated [12]. It would be of interest to learn whether the 43 000 mol. wt polypeptide [12] is identical to the 45 000 mol. wt polypeptide which we find has an ATP-binding site. We believe our results suggest that this polypeptide could be a membrane protein kinase or phosphatase involved in the regulation of AChR phosphorylation at the post-synaptic membrane.

Acknowledgements

We thank Dr Stella J. Jeng for the synthesis of arylazido-β-alanyl ATP and Mr Giampiero Bandini for excellent technical assistance. Financial support by the Deutsche Forschungsgemeinschaft, SFB 138, and NATO research grant no. 1374 is gratefully acknowledged. R.J.G. was on sabbatical leave in Konstanz.

References

[1] Gordon, A. S., Davis, C. G., Milfay, D. and Diamond, I. (1977) Nature 267, 539–540.
[2] Teichberg, I., Sobel, A. and Changeux, J. P. (1977) Nature 267, 540–542.
[3] Gordon, A. S., Davis, C. G. and Diamond, I. (1977) Proc. Natl. Acad. Sci. USA 74, 263–267.
[4] Gordon, A. S., Milfay, D., Davis, C. G. and Diamond, I. (1979) Biochem. Biophys. Res. Commun. 87, 876–883.
[5] Teichberg, I. and Changeux, J. P. (1977) FEBS Lett. 74, 71–76.
[6] Schiebler, W. and Hucho, F. (1978) Eur. J. Biochem. 85, 55–63.
[7] Jeng, S. T. and Guillory, R. T. (1975) Supramol. Biol. 3, 448–468.
[8] Laemmli, U. K. (1970) Nature 227, 680–685.
[9] Jean, D. H., Albers, R. W. and Koval, G. J. (1975) J. Biol. Chem. 250, 1035–1040.
[10] Giotta, G. J. (1975) J. Biol. Chem. 250, 5159–5164.
[11] Pitts, B. J. R. and Schwartz, A. (1975) Biochim. Biophys. Acta 401, 184–195.
[12] Sobel, A., Heidmann, T., Hofler, J. and Changeux, J. P. (1978) Proc. Natl. Acad. Sci. USA 75, 510–514.