EVIDENCE FOR PROTEIN PHOSPHORYLATION AND DEPHOSPHORYLATION IN MEMBRANE FRAGMENTS ISOLATED FROM THE ELECTRIC ORGAN OF *ELECTROPHORUS ELECTRICUS*

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1. Introduction

In a previous communication [1] it was shown that in crude detergent extracts of membrane fragments from *Electrophorus electricus* electric organ, the acetylcholine receptor protein (AChR) is present under two forms with different isoelectric points. A catalytic interconversion between these two forms was also achieved by incubating in vitro the crude membrane extract in the presence of 100 mM NaCl or 100 mM NaF. It was then proposed that the difference in isoelectric points between the two forms of AChR and their catalytic interconversion could result from phosphorylation–dephosphorylation reactions catalyzed by protein kinases and phosphoprotein phosphatases. At that time, however, this interpretation was not substantiated by evidence for the existence of a phosphorylating system in the crude detergent extract of membrane fragments.

In this letter, we show that endogeneous protein kinase and phosphoprotein phosphatase activities are indeed present in membrane fragments from *E. electricus* electric tissue. Furthermore, polyacrylamide gel electrophoresis in sodium dodecyl sulfate of the labeled crude extract followed by autoradiography indicates that polypeptides of molecular weight 50 000, 43 000, 40 000 and 32 000 are the substrates for the endogeneous protein kinase activity.

Abbreviations: AChR acetylcholine receptor protein, PMFS para-methylsulfonyl fluoride, SDS sodium dodecylsulfate

2. Materials and methods

2.1. *Preparation of membrane fragments*

Frozen or fresh electric tissue (40 g) from *Electrophorus electricus* were homogenized into 80 ml of 0.2 M sucrose in PNM (10^{-4} M para-methyl sulfonyl fluoride (PMFS) a protease inhibitor from Sigma Inc., 0.02% NaN_3, 0.014 M 2-mercaptoethanol in H_2O pH 6.2) for 3 min at 0°C using a Virtis homogenizer at medium speed. The homogenate was centrifuged for 10 min at 1500 × *g* at 4°C. The supernatant was collected, filtered through cheese cloth and centrifuged for 60 min at 13 000 rev/min in a Jouan centrifuge using a JA21 rotor at 4°C. The pellet resuspended into 4 ml of PNM was then applied on top of a 30 ml 45–20% linear sucrose gradient in PNM and centrifuged at 4°C in a SW27 rotor for 270 min at 25 000 rev/min. The gradient was collected in 2 ml fractions.

2.2. *Preparation of crude detergent extract of membrane fragments*

Crude Triton X-100 extracts of membrane fragments (S_2) were prepared as described in [1].

2.3. *Protein kinase assay*

Protein kinase activity was measured in the following standard assay mixture: 100 mM Tris, 10^{-3} M ouabain, 10 mM $MnCl_2$, 100 mM NaF, 4 μM [γ-^{32}P]-ATP (~ 16 Ci/mM purchased from Amersham) at pH 7.3. The total assay mixture was of 100 μl and contained 50 μl of the aliquot to be assayed. The incubation was carried out at room temperature for 3 min and the reaction was stopped by addition of 30 μl of 15 mM cold ATP in H_2O; 110 μl of the

mixture were then applied on a Whatman 3 MM disk filter of 25 mm diameter and immediately dipped into a beaker containing 400 ml of 5% perchloric acid. The filter disks were gently stirred for 15 min and placed into a fresh 400 ml 5% solution of perchloric acid. This operation was repeated three times. The disk-filters were washed with 90% alcohol, then with dried ether and counted in Toluene-POPOP solution.

2.4. *Assays*

Proteins were estimated by the method of Lowry et al. [2] using bovine serum albumin as the standard. Acetylcholinesterase activity was assayed with acetylthiocholine as substrate by the method of Ellman [3]. Acetylcholine receptor was monitored in the sucrose gradient by adding a small amount of ^{3}H-labelled α-toxin from *Naja nigricollis* [4] to the membrane fragments prior to the centrifugation as described by Olsen et al. [5].

2.5. *Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate*

SDS–gel electrophoresis was carried out on slab-gels of 1.5 mm width following the method of Laemmli [6] modified by Anderson and Gesteland [7]. The gels were stained for protein with Coomassie Blue, dried and placed in contact with Kodak Kodirex film and the film developed 3–7 days later. Scanning of the autoradiographs was carried out using a Vernon scanner. The following proteins were used as markers for molecular weight determinations: chymotrypsin, aldolase, albumin, catalase, bovine serum albumin.

3. Results

When membrane fragments isolated from the electric organ of *E. electricus* are incubated in the presence of [γ-^{32}P]ATP, Mg^{2+} or Mn^{2+} ions, an incorporation of phosphate groups into endogenous proteins takes place (table 1). This incorporation can

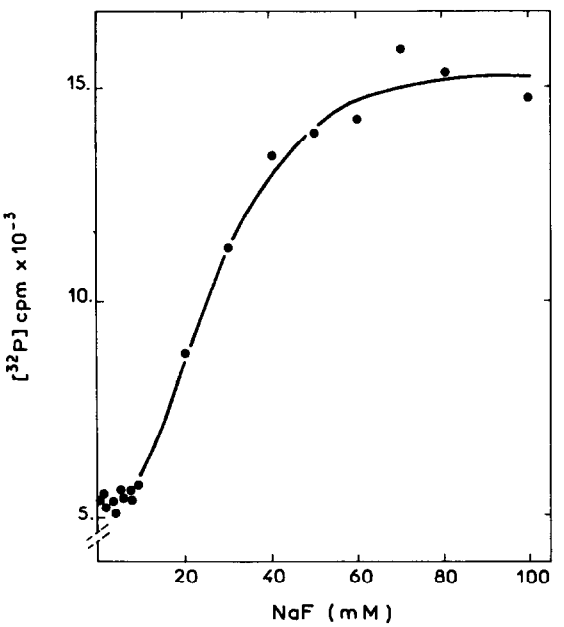

Fig.1. Stimulation by NaF of the incorporation of [^{32}P]-phosphate groups in endogeneous proteins of S_2. Assay buffer, pH 7.3, 39 mM Tris, 3.9×10^{-4} M ouabain, 7.8 mM $MnCl_2$. 1.31 μM [γ-^{32}P]ATP (14.2 Ci/mM). The incubation mixture at each NaF concentration contains in 100 μl, 2.46×10^{6} cpm of [γ-^{32}P]ATP.

Table 1

Level of incorporation of [^{32}P]phosphate groups into endogenous proteins from a crude Triton X-100 extract of membrane fragments of *E. electricus*

Complete system: 100 mM Tris, pH 7.3, 10^{-3} M ouabain, 10 mM $MnCl_2$, 100 NaF, 4 μM [γ-^{32}P]ATP	100
− $MnCl_2$	10
− $MnCl_2$ + 10 mM $MgCl_2$	82
− NaF + 100 mM NaCl	25
+ 10^{-6} M cyclic AMP	98
+ 15 mM ATP	< 1

The incubation was carried out in a total volume of 100 μl at 22°C for 30 min. In a typical experiment, the total radioactivity from [γ-^{32}P]ATP was 6×10^{5} cpm. The total incorporated was 4.4×10^{4} cpm, i.e., 7.3%. The blank (complete system + 15 mM ATP added at the beginning of the incubation) was 400 cpm.

be visualized by the presence of labeled polypeptides that can be separated by polyacrylamide gel electrophoresis in SDS and analyzed by autoradiography. The protein kinase activity requires the presence of divalent cations and has an apparent K_m for ATP of 4 μM. NaF strongly stimulated the incorporation of ^{32}P whereas adenosine 3',5'-monophosphate (cyclic AMP) has no effect (table 1).

Figure 1 illustrates the stimulation by increasing concentrations of NaF of the incorporation of [^{32}P]-phosphate groups into endogenous proteins from a crude Triton X-100 extract of the same membrane fragments. The concentration–effect curve in the presence of 10 mM Mn^{2+} (Mn^{2+} is used instead of Mg^{2+} because of the high solubility product of MnF_2) has a slight sigmoid shape and half maximal stimulation occurs in the presence of 25 mM NaF, the maximal stimulation being reached at 70 mM NaF. Without ambiguity, a protein kinase is present in membrane fragments from *E. electricus* electric organ but the effect of NaF may result either from a direct effect on the protein kinase or from an inhibition of a phosphoprotein phosphatase [8].

To investigate this problem, the kinetics of ^{32}P-incorporation into endogenous proteins from S_2 was followed at 22°C in the presence of either 100 mM

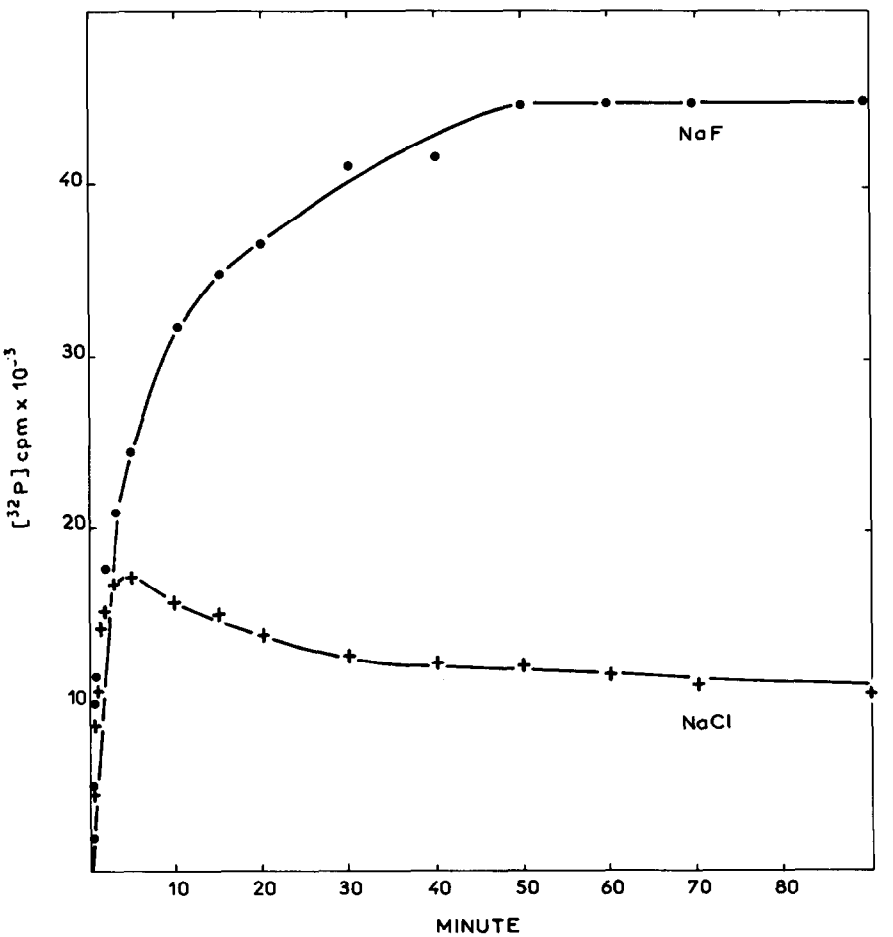

Fig.2. Kinetics of incorporation of [γ-^{32}P]phosphate groups into endogenous proteins of S_2. In the presence of 100 mM NaF (• — •). In the presence of 100 mM NaCl (+ – +). Assay buffer, pH 7.3, 67 mM Tris, 6.7 × 10^{-4} M ouabain, 10 mM $MnCl_2$, 10 μM [γ-^{32}P]ATP (13.7 Ci/mM).

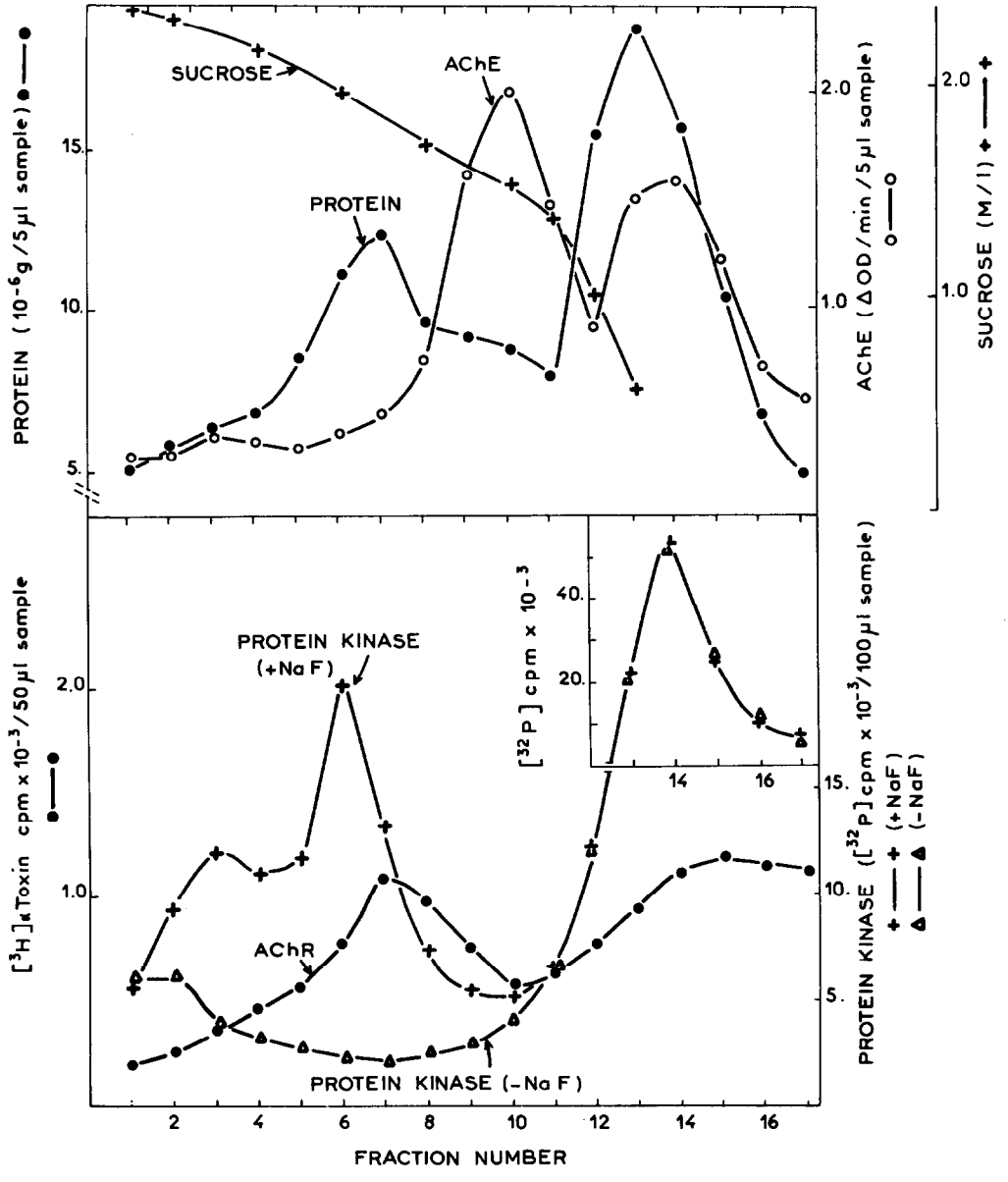

Fig.3. Distribution of acetylcholine receptor AChR, acetylcholinesterase AChE, protein kinase (+ NaF), phosphoprotein phosphatase (Protein kinase–NaF) and total protein in a sucrose gradient after ultracentrifugation of a crude membrane preparation of *Electrophorus electricus* electric organ with ^{3}H-labeled α-toxin. The assay conditions are described in Materials and methods. The specific activity of fraction No. 7 was 80 nM of toxin binding sites per gram protein.

NaF or 100 mM NaCl. In the presence of NaF, the incorporation of ^{32}P is linear as a function of time for 3 min and reaches a plateau after 50 min. In the presence of NaCl however, a composite effect is observed. For the first 3 min, the incorporation is linear but then the level of incorporation slowly decreases and reaches a plateau corresponding to an incorporation of approx. 25% of that found in the presence of NaF. The most simple interpretation of this result is that a phosphoprotein phosphatase activity competes with that of the protein kinase. In order to determine whether the activity of protein kinase and phosphoprotein phosphatase were also present in the native membrane fragments containing the AChR, these activities were followed in membrane fractions isolated by sucrose gradient centrifugation. The activity of the protein kinase was measured in the presence of NaF whereas the phosphoprotein phosphatase was determined by its competing action on protein kinase in the absence of NaF. Figure 3 shows the presence of a protein kinase in a membrane subpopulation migrating at a sucrose density higher than that of the AChR containing membranes labeled by *N. nigricollis* ^{3}H-labeled α-toxin. The phosphoprotein phosphatase, however, seems to migrate at the same density as that of AChR. A very high activity of protein kinase is also present in membranes of a lower density (fractions 13–15) which are devoid of phosphoprotein phosphatase as well as of AChR. The counts of tritium observed in fractions 12–17 are due to denatured ^{3}H-labeled α-toxin present in the particular batch of toxin used. Acetylcholinesterase migrated with membrane fragments of intermediate density.

In order to identify the substrate of the protein kinase activity, the polypeptides labeled in an incubation mixture with [γ-^{32}P]ATP were analyzed by polyacrylamide gel electrophoresis in SDS. Figure 4 shows a typical autoradiograph obtained for a S_2 fraction labelled with [γ-^{32}P]ATP. Mainly four polypeptides of 50 000, 43 000, 40 000 and 32 000 daltons are substrates for the endogeneous protein kinase. Interestingly, if such a S_2 fraction after labeling with [γ-^{32}P]ATP is passed through a column of erabutoxin b coupled to Sepharose 4 B eluted with 1% SDS and further analyzed by SDS–polyacrylamide gel electrophoresis and autoradiography, an identical profile of radioactive polypeptides is obtained.

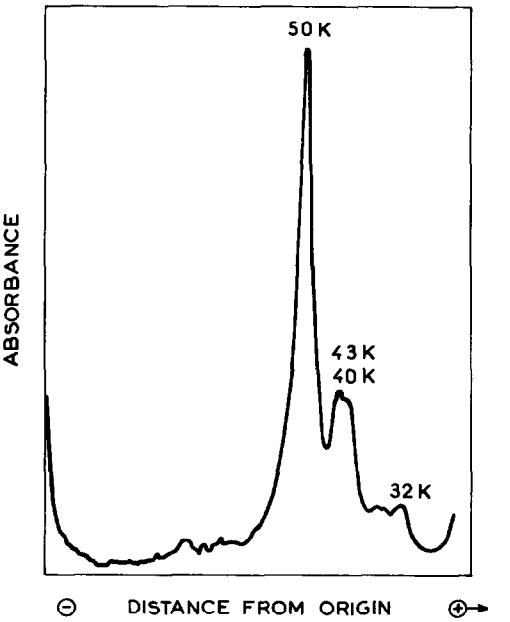

Fig.4. Polyacrylamide gel electrophoresis in dodecyl sulfate of the products of phosphorylation of S_2 by an endogeneous protein kinase. The incubation conditions were those described in fig.1 and the time of incubation was 30 min.

4. Discussion

Neural tissue and in particular brain synaptic membranes [9] contain protein kinases and phosphoprotein phosphatases which are thought to play a role in synaptic transmission. Membrane fragments from the electric organ of *E. electricus* display the same feature as they also possess a phosphorylating system. Endogenous proteins serve as substrates for protein kinase and phosphoprotein phosphatase. The activity of protein kinase can be readily observed upon addition of divalent cations such as Mg^{2+} or Mn^{2+} and ATP. The protein kinase is not activated by cyclic AMP as observed in many systems [9]. This may result from the conditions of our isolation procedures. The presence of a phosphoprotein phosphatase, however, is inferred from the stimulatory effect of NaF on the protein kinase activity. Both the sigmoidal response of the protein kinase with increasing NaF concentration and the kinetics of incorporation of [γ-^{32}P]phosphate groups in the presence and absence of NaF points out to the presence of a phosphoprotein

phosphatase whose activity can be totally inhibited by 70 mM NaF. These enzyme activities are present in membrane fragments which migrate in sucrose gradient in discrete subpopulations. At this stage, it is not clear whether these enzymes belong to the excitable AChR rich membrane and are redistributed during the preparation and isolation procedures. By analogy with acetylcholinesterase, which is known to be attached to the subsynaptic excitable membrane, it is also possible that the phosphorylating system is bound to the excitable membrane without being an integral part of it. SDS–polyacrylamide gel analysis shows that essentially four polypeptides of 50 000, 43 000, 40 000 and 32 000 daltons are phosphorylated in S_2. Although the purified AChR from *Electrophorus* is thought to be composed of polypeptides of 48 000 ± 3000 and 43 000 ± 3000 daltons, with the appearance upon aging of a polypeptide of 35 000 daltons [10] it is not possible to draw from this analogy any definite conclusion as to the AChR serving as a substrate for protein kinase. However, preliminary experiments (to be published) indicate that indeed the AChR protein can be phosphorylated.

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