Acetylcholine receptor-rich membranes contain an endogenous protease regulated by peripheral membrane protein

Jan Verdenhalven, Giampiero Bandini and Ferdinand Hucho

Freie Universität Berlin, Institut für Biochemie, Fabeckstr. 34-36, 1000 Berlin 33, Germany

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1. INTRODUCTION

Postsynaptic receptor density is under thorough control and there is evidence that in the neuromuscular endplate the concentration of nicotinic acetylcholine receptors (AChR) is determined not only by the rate of biosynthesis but also by the degradation rate [1]: half-life of extrasynaptic AChR is ~7 h; subsynaptic receptors in contrast are turned over much more slowly (half-life 22 days, [1]; myasthenia gravis sera [2] as well as purified anti-AChR antibodies [3] enhance AChR degradation. We therefore investigated the proteolytic activities possibly responsible for this degradation and found 2 different types of proteases [4,5]; one class, probably of lysosomal origin, removable by normal biochemical methods, another endogenous to purified membrane fractions rich in AChR. Here, we report the observation that the latter is regulated by a factor removable by alkaline treatment of the AChR-membranes according to [6]. This factor may be identical to the major polypeptide removed by this method, a protein of M_r 43 000, a peripheral membrane protein named α' [7] and considered to be a stabilising factor of the postsynaptic membrane [8,9]. A more detailed preliminary description of the membrane-bound protease concerning its ion and thiol-dependence has been published [10].

2. MATERIALS AND METHODS

These experiments were done with the model system for neuromuscular endplates, the electric organ of the ray *Torpedo californica*. We isolated membranes especially rich in AChR by the method in [11], as modified [12]. EDTA was present in the first homogenisation and washing steps, because it was shown that EDTA, and more specifically EGTA, preserves the subunit composition of the AChR [13].

A quantitative assay of the receptor-membrane protease is not yet available because we did not find an exogenous substrate. Instead protease activity was estimated qualitatively from changes in the band pattern of its apparent endogenous substrate, the AChR, as visualised by SDS–polyacrylamide gel electrophoresis.

Proteolysis was accomplished by incubating AChR-rich membranes in Ringer's solution (pH 8.4) for the time indicated in the figure legends, which also contain further details of the incubation conditions. SDS–polyacrylamide gel electrophoresis was performed on 10% polyacrylamide gels as in [17].

3. RESULTS AND DISCUSSION

Fig.1 shows that proteolysis occurs even in highly purified AChR-rich membranes showing predominantly the characteristic α,α',β,γ,δ band-pattern on stained SDS–polyacrylamide gels. We have several indications that this proteolytic activity is distinct from lysosomal proteases in its pH-dependence (active only at pH > 8), its insensitivity against inhibitors of serine-proteases (PMSF), and its copurification with AChR-binding activity [10]. Our most purified receptor-rich membranes after sucrose density-gradient centrifugation still contain this proteolytic activity. Treatment of these

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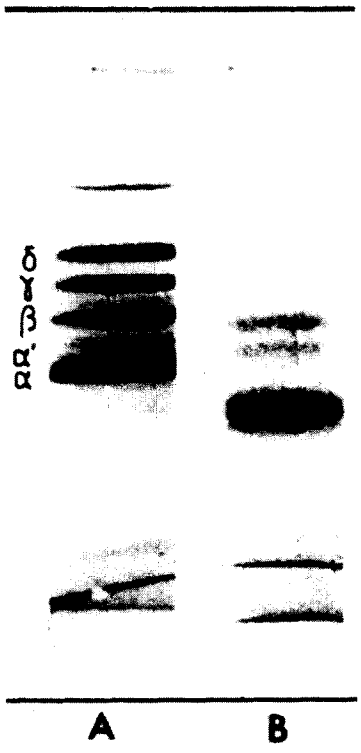

Fig.1. Endogenous protease activity in AChR-rich membranes: (A) control, 10% SDS–polyacrylamide gel electrophoresis of AChR-rich membranes showing the characteristic integral membrane proteins α, β, γ, δ, and the peripheral protein α'; (B) same preparation after 3 days incubation at pH 8.4 and room temperature.

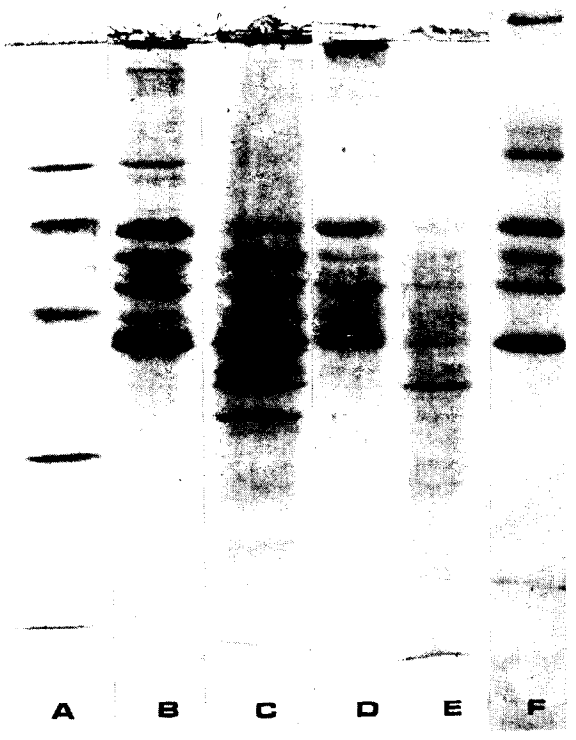

Fig.2. Action of endogenous protease on AChR-rich membrane fragments extracted at pH 11. Receptor-rich membrane fragments prepared from *Torpedo californica* electric organ (track B) were treated at alkaline pH as in [6] (track F): (A) calibration standards (from top to bottom: phosphorylase, bovine serum albumin, carbonic anhydrase, trypsin inhibitor); (B) AChR incubated for 3 days at pH 8.4 at 0°C; (C) AChR-rich membrane fragments extracted at pH 11, after 3 days incubation at 0°C and at pH 8.4; (D) same as (C), but after readdition of the membrane components extracted at pH 11. Readdition of the extracted components was achieved by neutralising the pH 11 mixture [6] without removing the supernatant. This makes certain that the peripheral membrane components are present in the correct stoichiometry. (E) AChR-rich membrane fragments without pH 11 treatment after 3 days incubation at 36°C. Slab gel SDS–polyacrylamide gel electrophoresis according to [17] on 10% polyacrylamide.

membranes at alkaline pH removes considerable amounts of supposedly peripheral protein, most conspicuously the α' polypeptide [6]. Even this treatment does not remove the proteolytic activity (fig.2). On the contrary, after alkaline treatment proteolysis proceeds much faster (fig.2, cf. B,C). We do not yet know whether this effect is due to the removal of an inhibitor of the protease or a molecule protecting the receptor against proteolysis as postulated for the proteolytic degradation of AChR by exogenous proteases [15]. Fig.2D, also shows that readdition of the membrane components extracted at pH 11 decreases again the rate of proteolysis.

Among the substances extracted from the AChR-rich membranes at alkaline pH there is obviously a component inhibiting the endogenous protease or protecting the AChR against proteolysis. It is appealing to investigate whether this component is the α' peptide, which was shown in to be a group of closely related proteins [16].

Whatever the correct interpretation of these effects, they appear to prove that AChR-rich membranes contain an endogenous, membrane-bound

protease. This conclusion is supported by the observations that:

(i) The proteolytic activity copurifies with the receptor even through its high-salt washes and density gradient purification steps [10];

(ii) It cannot, like other peripheral proteins of the receptor-rich membranes, be removed by treatment at pH 11.

Further evidence [5,10] in preparation comes from the observation that this proteolytic activity does not digest soluble protease substrates like hemoglobin or casein and is inhibited by conditions favourable for lysosomal proteases (mild detergents, low pH).

Besides hemoglobin and casein, [^{14}C]cyanate-labeled hemoglobin and the following chromogenic protease substrates were tested: Hide powder Azure, Mα-benzoyl–L-Arg–β-naphthylamide, L-Leu–β-naphthylamide, Glu–Gly–Gly–Phe–β-naphthylamide, N-CBZ–Ala–Arg–Arg–4-methoxy-β-naphthylamide. With Leu–β-naphthylamide only peptidase activity was observed, but this activity did not show the characteristic calcium dependence. It was not blocked by 2 mM EDTA and was present in the AChR-rich fractions of the sucrose density gradient in traces only but in fairly large quantity on top of the gradient. It appears to be due to a leucineaminopeptidase and is inhibited by puromycin.

We can only speculate as to the physiological significance of the observed proteolytic activity. It may play a role in receptor turnover by nicking the protein as a first step of its internalisation and degradation. It is appealing to presume that the pH 11-extractable inhibitor of this membrane-bound protease is located specifically in the postsynaptic membrane thereby slowing down the turnover of subsynaptic as compared to extrasynaptic receptors [1].

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