MODIFICATION OF ACETYLCHOLINE RECEPTOR: CHEMICAL AND IMMUNOLOGICAL CHARACTERIZATION OF POLYALANYL ACETYLCHOLINE RECEPTOR

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1. Introduction

The pharmacological and immunological characteristics of the nicotinic acetylcholine receptor (AChR) in its membrane as well as in its aqueous and detergent solubilized forms have been studied in details [1–4]. All these receptor preparations express similar binding properties to cholinergic ligands and they can all induce experimental autoimmune myasthenia gravis (EAMG) [5–7].

Molecular modifications of proteins are a useful approach for elucidating the relationship between their structure and biological function. We have studied the effect of denaturation and of proteolytic degradation of AChR on its biological activity. Such studies demonstrated that a denatured AChR preparation obtained by reduction and carboxymethylation of AChR in 6 M guanidine (RCM-AChR) is devoid of both abilities to bind cholinergic ligands and to induce EAMG [8], but had a suppressive effect on EAMG [9]. On the other hand tryptic digestion of AChR did not change the pharmacological specificity and pathological myasthenic activity of the receptor molecule although it resulted in a simpler and smaller molecule composed of one type of subunit (M_r ~27 000), lower in molecular weight from all the four subunits present in the intact receptor [10].

Polyalanylation of proteins is a specific mild chemical modification achieved by attaching poly(D,L-Ala) side chains to free amino groups on proteins, by polymerization of *N*-carboxy-D,L-alanine anhydride in aqueous media. The reason for using D,L-Ala anhydride for polymerization is that poly(D,L-Ala) is water soluble whereas either poly(L-Ala) or poly(D-Ala) are not soluble [11]. Polyalanylation of enzymes did not change their enzymic activity [12]; also in some cases the attachment of D,L-Ala peptide chains to proteins has increased their solubility [13].

We report here on the preparation and characterization of poly(D,L-Ala) AChR (PA-AChR). Alanylation of AChR did not affect significantly the cholinergic binding properties of the receptor whereas it abolished its myasthenic activity. Thus, by this modification the pharmacologic and myasthenic activities of the receptor have been dissociated.

2. Materials and methods

AChR was isolated from the electric organ of *Torpedo californica* and was purified as in [6]. Radiolabelling of proteins with ^{125}I was performed by the chloramine-T method [14]. Specific binding of α-bungarotoxin (α-Bgt) to AChR and to PA-AChR was determined by measuring the amount of ^{125}I-α-BgT which coprecipitates with the receptor in 35% saturated ammonium sulfate [6].

2.1. *Preparation of PA-AChR*

PA-AChR was prepared by reacting AChR with *N*-carboxy-D,L-alanine anhydride [15] in a procedure similar to that described for alanylation of other proteins [11,13]. AChR (1 mg/ml phosphate buffered saline (PBS)) was reacted in the cold with *N*-carboxy-D,L-alanine anhydride dissolved in anhydrous dioxane (4 mg anhydride/mg protein; final conc. dioxane was ≤10%). The mixture was kept for 24 h at 4°C with gentle stirring and was then dialyzed for 3 days against several changes of PBS. The dialysate was centrifuged to remove any highly aggregated material. For control, AChR alone or AChR in 10% dioxane were treated in a similar manner.

2.2. *Biochemical analysis*

Amino acid composition was determined in a Beckman automatic amino acid analyzer, Model 121. Sedimentation coefficients were determined by sucrose gradient centrifugation and by analytical ultracentrifugation [6]. Inhibition constants for cholinergic ligands were determined by measuring their effect on the initial rate of formation of the complex between ^{125}I-α-Bgt and the tested receptor preparation, by a procedure similar to that in [6,16,17].

2.3. *Immunization and immunological assays*

Rabbits were immunized 2 or 3 times, each time with 100 μg PA-AChR (in 1 ml) emulsified with equal volumes of complete Freund's adjuvant. Injections were given into the footpads and intradermally at multiple sites at 30 day intervals. For comparison, other rabbits were injected once or at most twice with AChR. Antisera were analysed by immunodiffusion and radioimmunoassay. Binding and inhibition experiments were performed by radioimmunoassay procedures similar to those in [18].

3. Results

3.1. *Biochemical characterization of PA-AChR*

Amino acid analysis of PA-AChR demonstrates that an av. 5 Ala residues were attached/1 Lys residue in the receptor molecule. This corresponds to a weight increase of ~18% based on a content of 6 mol % lysines in AChR [6]. In order to verify that the alanine residues were covalently bound to AChR we tested whether PA-AChR can be adsorbed to an antibody column of anti-polyalanine antibodies. Radioiodinated PA-AChR was adsorbed to a Sepharose adsorbent of anti-poly(D,L-Ala)-ribonuclease immunoglobulins, whereas ^{125}I-AChR was not adsorbed at all.

Table 1
I_{50} values for AChR and PA-AChR

Ligand	I_{50} (M)	
	AChR	PA-AChR
d-Tubocurarine	9.0×10^{-7}	5.0×10^{-6}
Carbamylcholine	3.0×10^{-5}	2.9×10^{-4}
α-Bungarotoxin	2.0×10^{-10}	3.2×10^{-9}
Atropine	2.2×10^{-4}	1.0×10^{-3}

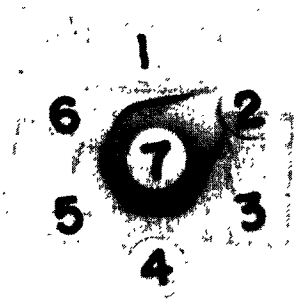

Fig.1. Immunodiffusion of anti-AChR serum (well 7) with PA-AChR (wells 1,5), polyalanyl BSA (well 2), AChR (wells 3,6) and AChR treated with 10% dioxane (well 4).

The adsorbed PA-AChR could be eluted from the adsorbent by means of poly(D,L-Ala) bovine serum albumin (4 mg/ml). This experiment proves that the added Ala residues were covalently attached to AChR.

Sucrose density gradient centrifugation of PA-AChR (complexed with ^{125}I-α-Bgt), demonstrated a sedimentation coefficient of 9 S. On analytical ultracentrifugation PA-AChR revealed one component of 9.3 S whereas with the unmodified receptor two protein components of 9 S and 13.4 S were observed, as in [6].

Alanylation of AChR did not affect significantly the capacity to bind α-Bgt. AChR and PA-AChR bound, respectively, 8000 and 6600 pmol α-Bgt/mg receptor. The control preparation of AChR which was reacted with 10% dioxane in the absence of the anhydride had also a slightly decreased specific activity (6700 pmol α-Bgt/mg). When the pharmacologic specificities of PA-AChR and AChR were compared by measuring the inhibition of the binding of ^{125}I-α-Bgt to PA-AChR and AChR by several cholinergic ligands, slightly higher I_{50} values (5–10-times higher) were obtained for the modified receptor than those obtained for AChR (table 1).

3.2. *Immunological characterization of PA-AChR*

The immunogenicity and antigenicity of PA-AChR were tested and compared with those of AChR. The binding of PA-AChR to anti-AChR serum is identical to the binding of AChR to this antiserum. This was observed by immunodiffusion (fig.1) and radioimmunoassay experiments. Similar binding of ^{125}I-AChR and ^{125}I-PA-AChR to anti-AChR serum was observed (fig.2a). Moreover, unlabelled AChR and

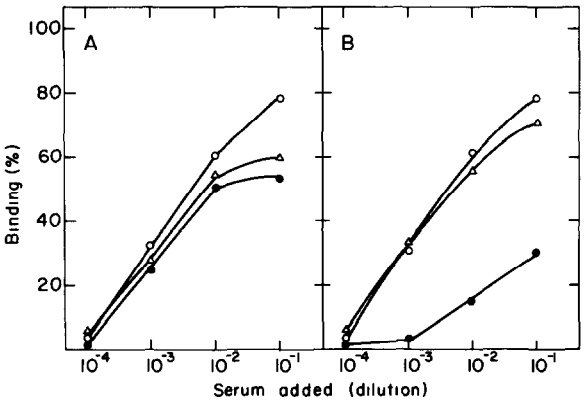

Fig.2. Binding of ^{125}I-AChR (•), ^{125}I-PA-AChR (○) and of ^{125}I-RCM-AChR (△) to anti-AChR serum (A) and to anti-PA-AChR serum (B).

PA-AChR inhibit to a similar extent the binding reactions of ^{125}I-AChR and ^{125}I-PA-AChR to anti-AChR serum (fig.3a,b).

As an immunogen PA-AChR appears to be quite different from AChR. Most of the antibodies in PA-AChR injected rabbits are directed against the polyalanine side chains (fig.2b,3c). There is only a partial cross reactivity between anti-PA-AChR serum and AChR. It should be noted also that the binding of anti-PA-AChR antibodies to a denatured receptor preparation (reduced carboxymethylated AChR, RCM-AChR [8]) is better than their binding to AChR (fig.2b).

Antibodies to AChR were shown to block the physiological activity of AChR [5,19,20] and to inhibit the binding of α-Bgt to the receptor [6]. However, anti-PA-AChR antibodies do not inhibit the binding of α-Bgt to AChR. This is in agreement with the finding that these antibodies bind poorly to AChR and that this reactivity is probably via non-structural antigenic determinants present on the

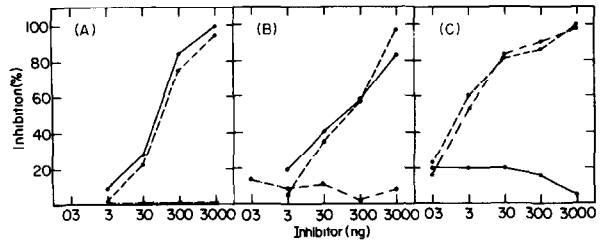

Fig.3. Inhibition of the binding of: (A) ^{125}I-AChR to anti-AChR-serum; (B) ^{125}I-PA-AChR to anti-AChR serum; (C) ^{125}I-PA-AChR to anti-PA-AChR serum by AChR (——), PA-AChR (– –) and PA-BSA (polyalanyl-BSA) (–·–).

denatured receptor (RCM-AChR). Anti-RCM-AChR antibodies also do not block the binding of α-Bgt to AChR as reported [8].

Repeated immunizations of rabbits with PA-AChR did not result in any clinical symptoms of experimental autoimmune myasthenia gravis (EAMG). However, 3 preimmunizations with PA-AChR before administration of AChR had an effect on the onset of EAMG, indicating the immunological relationship between the two receptor derivatives. In 5 out of 10 rabbits, preimmunization with PA-AChR resulted in a protecting effect expressed by a delay in the onset or prevention of EAMG, whereas in another 3 there seemed to be a facilitating effect and EAMG appeared 5–10 days following the first AChR administration.

4. Discussion

Chemical modification of AChR provides a valuable tool for identifying and analysing the structure of the biologically active site(s) of the molecule. These studies demonstrate that the myasthenic activity of the receptor is not necessarily identical to its pharmacological activity. PA-AChR which retains the cholinergic binding activity of AChR does not induce myasthenia. In two other AChR derivatives studied in our laboratory both functions were expressed similarly. Denatured AChR (RCM-AChR) was shown to be devoid of both cholinergic and myasthenic activities [8,9] whereas in trypsinated AChR (T-AChR) both functions were not affected [10].

The finding that polyalanylation of lysines does not interfere with the binding properties of AChR may imply either that lysine residues (or other free amino groups) do not participate in the cholinergic binding site or alternatively that these particular residues in the active site were not susceptible to the modification.

The immunogenicity of PA-AChR is markedly different from that of AChR. The polyalanine side chains appear to be immunopotent and the majority of the antibodies in anti-PA-AChR is directed against the polyalanine side chains (fig.3c). However, there are also some anti-AChR antibodies; these latter antibodies are probably directed against non structural determinants of AChR, which are not effected by denaturation, as they react preferably with RCM-AChR (fig.2). This specificity [9] is also compatible with the finding that PA-AChR is not myasthenic.

Analysis of the pharmacologic, antigenic and immunogenic specificities of PA-AChR contribute towards the elucidation of the structure of the biological active sites of AChR. The non-pathogenicity of PA-AChR along with its antigenic specificity renders it also a possible candidate for regulating EAMG.

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