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LOCALIZATION OF A HIGHLY IMMUNOGENIC REGION ON THE ACETYLCHOLINE RECEPTOR α-SUBUNIT

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Antibodies to synthetic peptides were employed in order to map domains on the α-subunit of the acetylcholine receptor to which several monoclonal antibodies are directed. Five peptides corresponding to residues 1-20, 126-143, 169-181, 330-340 and 351-368 of the receptor α-subunit were synthesized and antibodies against them were elicited. The anti-peptide antibodies were employed along with the monoclonal antibodies to identify fragments of S. aureus V8 protease digested- α-subunit in immunoblotting experiments. Our results demonstrate that a highly immunogenic region of the α-subunit is located on a carboxy-terminal 14 kDa portion of the α-subunit. This region also seems to undergo antigenic changes during muscle development. A monoclonal antibody directed against the cholinergic binding site of the acetylcholine receptor reacted with an 18 kDa segment of the α-subunit which bound α-bungarotoxin as well as antibodies directed against peptide 169-181.

The nicotinic acetylcholine receptor (AChR) is a complexed multideterminant protein, and consists of four subunits α,β,γ,δ, present in a stoichiometry of 2:1:1:1 respectively. This protein encompases a variety of functional domains such as the ligand binding site, an ion channel, phosphorylation sites, glycosylation sites and immunogenic regions which may be involved in the immune response to AChR and in myasthenia gravis (see 1-5 for reviews). In order to analyze these domains in the AChR and to delineate the immune response to AChR in myasthenia we have previously prepared a library of monoclonal antibodies (mcAbs) elicited against Torpedo AChR (6-8). These mcAbs were employed as probes to study the function, metabolism and pathogenicity of the AChR (8-10).

Mapping the antigenic determinants to which the mcAbs are directed is feasible, by employing antibodies against synthetic peptides corresponding to selected regions on the AChR molecule. Thus, it may be possible to pinpoint the region responsible for a certain function. This approach has been employed in our laboratory in order to localize the ligand binding site of the receptor

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(11). Other groups have utilized antibodies to synthetic peptides for analyzing the transmembrane orientation of the AChR molecule (12-14), and its main immunogenic region (15,16).

In this study we have employed antibodies to synthetic peptides of the AChR α-subunit in order to localize the antigenic determinants towards which some mcAbs are directed. Our results demonstrate that the majority of mcAbs are directed towards a 14 kDa carboxy-terminal region of the α-subunit, indicating that a highly immunogenic domain resides there. We also demonstrate that a mcAb (5.5) directed against the receptor ligand binding site (7), binds an 18 kDA fragment of the α-subunit which also recognizes antibodies against peptide 169-181.

MATERIALS AND METHODS

AChR purification: AChR purification from Torpedo Californica was performed as previously described (17).

Production of mcAbs: A library of mcAbs has been elicited against Torpedo AChR in two different hybridizations as previously reported (6,7).

Peptide synthesis and conjugation: Synthesis of peptides corresponding to residues 1-20 and 126-43 of the AChR α-subunit was described elsewhere (11,18). Peptides coresponding to residues 169-181, 330-340 and 351-368 of Torpedo AChR α-subunit were synthesized similarly by the Merrifield solid phase procedure (19) and their amino acid ratios corresponded well with that expected. The peptides were conjugated to bovine serum albumin (BSA; Sigma) using 1-ethyl-3-3'-dimethylaminopropylcarbodiimide (Sigma) as the coupling reagent (20).

Blot analysis of the AChR and of the digested α-subunit: AChR was resolved to its subunits in polyacrylamide gel electrophoresis (PAGE), containing lithium deodecyl sulfate (LDS;21). Digestion of the α-subunit with S. aureus V8 protease was performed as previously described (11). Electrophoretic transfer of the resolved AChR subunits or the proteolytic fragments of the α-subunit, as well as toxin overlay and antibody overlay were performed as described (11,22). For antibody overlay the strips were incubated with a 1:100 dilution of the different antibodies in quenching buffer (except for mcAb 5.5 which was used at 1:75 dilutions). All antipeptide antisera were absorbed on a column of S. aureus V8 protease prior to use. After 5 washes in phosphate buffered saline (PBS), and one wash in PBS containing 0.5% triton X-100, the strips were incubated for 1 hr in ^{125}I-labeled Protein A or ^{125}I-labeled-goat anti mouse-immunoglobulins ($5x10^5$ cpm/ml). The strips were then washed as above, and autoradiographed.

RESULTS

Characterization of the monoclonal antibodies:

The subunit specificity of the monoclonal antibodies employed in this study was determined by the immunoblotting method (Fig. 1). All these mcAbs bound to

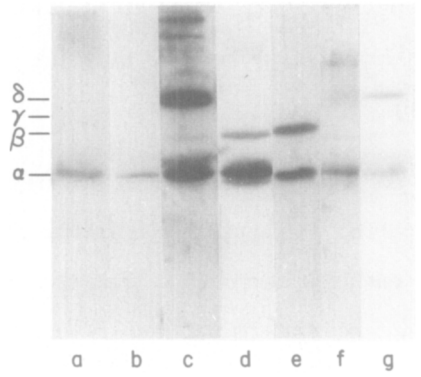


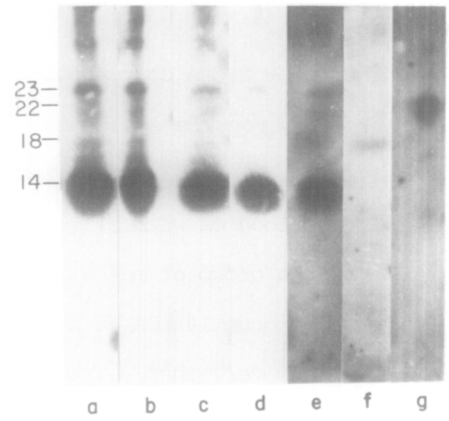
Fig. 1: Subunit specificity of mcAbs. Torpedo AChR was electrophoresed in a 10% plyacrylamide gel, blotted onto nitrocellulose filters and overlayed with the different mcAbs followed by ^{125}I-labeled-GaMIg. Overlay with a, mcAb 1.34; b, mcAb 5.14; c, mcAb 1.39; d, mcAb 1.22; e, mcAb 1.26; f, mcAb 5.5 and g, mcAb 5.46.

the α-subunit whereas some of them bound to additional subunits as well. Thus, monoclonal antibodies 1.34, 5.14 and 5.5 reacted predominatly with the α-subunit (Fig. 1a,b,f), mcAbs 1.39 and 5.46 cross reacted with the α and δ-subunits (Fig. 1c,g) and mcAbs 1.22 and 1.26 cross reacted with the α and β subunits (Fig. 1d,e). It should be noted that the immunoblotting conditions applied, enabled to determine the subunit specificity of mcAbs which previously failed to react with the isolated subunits in radioimmunoassay in solution (e.g. 5.5 and 5.46 (8)).

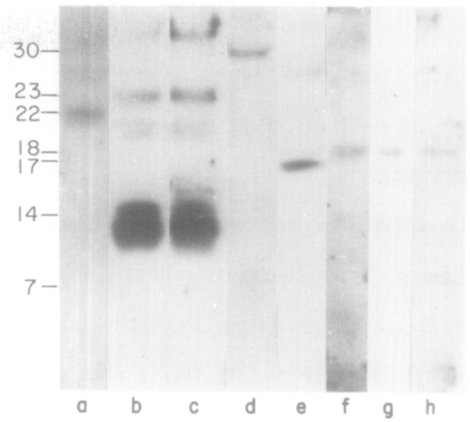
Mapping the antigenic determinants of the mcAbs:

For precise mapping of the antigenic determinants towards which the mcAbs are directed we applied blot analysis of S. aureus V8-proteolytic fragments of the α-subunit. The V8-proteolytic fragments were overlayed with the mcAbs, and with antibodies to synthetic peptides corresponding to sequences from the AChR α-subunit. All the anti-peptide antibodies employed cross-reacted with the AChR and bound specifically to its α-subunit (11, 18 and data not shown).

Blot analysis of seven mcAbs is shown in Fig. 2. Of these, mcAbs 1.22, 5.14, 1.34, 1.39 and 5.46, all reacted with the same proteolytic fragment of 14 kDa (Fig. 2a-e). On the other hand, mcAb 5.5 (Fig. 2f) and mcAb 1.26 (Fig. 2g) bound to an 18 kDa fragment and a 22 kDa fragment respectively. As can be



(2)



(3)

Fig. 2: Binding of mcAbs to proteolyzed α-subunit. The electrophoresed, proteolyzed α-subunit was blotted onto nitrocellulose filter and overlayed with the mcAbs followed by ^{125}I-labeled-GaMIg. Overlay with: a, mcAb 1.22; b, mcAb 5.14; c, mcAb 1.34; d, mcAb 1.39; e, mcAb 5.46; f, mcAb 5.5 and g, mcAb 1.26.

Fig. 3: Binding of mcAbs and antipeptide antibodies to proteolyzed α-subunit. McAbs and antipeptide antibodies were reacted with proteolyzed α-subunit followed by ^{125}I-labeled-GaMIg or ^{125}I-labeled-protein A as follows: a, mcAb 1.26; b, mcAb 5.14; c, anti-peptide 351-368 antibodies; d, anti-peptide 1-20 antibodies; e, anti-peptide 126-143 antibodies; f, mcAb 5.5; g, α-BTX and h, anti-peptide 169-181 antibodies.

seen in Fig. 3 the 14 kDa fragment which contains the antigenic sites of these five mcAbs (Fig. 2a-e and Fig. 3b) is also recognized by antibodies prepared against a synthetic peptide which corresponds to residues 351-368 of the α-subunit (Fig. 3c). Antibodies to peptide 1-20 and to peptide 126-143 of the α-subunit did not bind to this 14 kDa fragment, nor did ^{125}I-labeled-α-bungarotoxin (α-BTX) (Fig. 3d,e,g). This suggests that the 14 kDa fragment represents a segment from the carboxy-terminal portion of the α-subunit.

By employing our antipeptide antibodies we also set forth to map the site on the α-subunit towards which mcAb 5.5 (specific for the ligand binding site of AChR; 7) is directed. As seen in Fig. 3 (f-h) mcAb 5.5, α-BTX and antibodies to a synthetic peptide corresponding to residues 169-181 of the α-subunit, all bound to the same 18 kDa V8 fragment of the α-subunit. This is in accordance with our previous study (11) in which we have postulated that this 18 kDa ligand binding site, lies beyond residue Asp 152 of the α-subunit.

DISCUSSION

In this study we have employed antibodies to synthetic peptides corresponding to defined sequences of the AChR α-subunit, in order to map the antigenic determinants to which several anti AChR mcAbs are directed. Table 1 summarizes the binding specificities of the mcAbs and antipeptide antibodies with S. aureus V8 protease digested α-subunit. It was shown that a group of mcAbs (mcAbs 1.34, 1.22, 5.14, 1.39 and 5.46) are all directed at one 14 kDa V8 proteolytic fragment of the α-subunit. It should be noted however, that although these mcAbs reacted with the same fragment they are not directed against identical antigenic determinants as they differ in their subunit specificity (Fig. 1), as well as in other characteristics (8).

Blotting experiments have demonstrated that the 14 kDa fragment which binds many of the mcAbs also binds antibodies to a synthetic peptide corresponding to residues 351-368, and is therefore derived from the carboxy-terminal portion of the α-subunit. Also, mcAbs directed at this region as well as anti-peptide 351-368 antibodies, did not bind to trypsinated AChR (24) which is devoid of its carboxy-terminal portion (25). Since mcAbs would be expected to represent a statistical sampling of the antibody specificities obtained in anti-AChR sera, this domain is probably a highly immunogenic region of the receptor. This conclusion is strengthened by experiments in our laboratory which indicate that the main response in rabbits, two weeks following injection with AChR, is to this 14 kDa fragment (data not shown). It appears that the immunogenic

TABLE I: Immunological characterization of S. aureus V8 protease digested α-subunit

Fragment (kDa)	mcAb	Anti-peptide	Other markers
22	1.26	-	-
18	5.5	169-181	α-BTX (11,22)
17	-	126-143	Con A (23)
14	1.34,1.39,5.14 5.39,5.46	351-368	-

region to which most of our mcAbs are directed is different from the main immunogenic region (MIR) as defined by Tzartos and Lindstrom (26), who have mapped their MIR to the NH_2-terminal end of the α-subunit (16,27).

Unlike the above mentioned five monoclonal antibodies which bind the 14 kDa fragment, we found that mcAb 1.26 reacted with a 22 kDa fragment (Fig. 2g). This mcAb also reacted in a different manner than mcAbs 1.34, 1.22, 5.14 and 1.39 when tested with junctional and extrajunctional AChR (28). All the latter antibodies reacted preferentially, though not exclusively, with the extrajunctional form of the receptor, whereas mcAb 1.26 did not discriminate between junctional and extrajunctional receptors. In addition, antibodies against a sequence corresponding to residues 351-368 of the human and calf α-subunit reacted preferentially with the extrajunctional AChR from mouse muscle as compared to junctional AChR, in a similar manner to that displayed by mcAbs 1.22, 1.34, 5.14 and 1.39 (Neumann et al., in preparation). These observations may suggest that the 14 kDa fragment contains a region which undergoes developmental changes in the process of synaptogenesis.

McAb 5.5 which is directed towards the ligand binding site, bound to the same 18 kDa fragment which was also recognized by α-BTX and by antibodies to peptide 169-181 (Fig. 3 f,g,h). These results are in accordance with previous findings from our laboratory that mcAb 5.5 is specifically directed towards the actual binding site. As antipeptide 1-20, 126-143 and 351-368 antibodies did not bind to this 18 kDa toxin-binding fragment, we propose that it should map at a sequence present between residues 153 (the first S. aureus V8 protease cleavage point after Asn 142) and 350.

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