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The ligand binding domain of the nicotinic acetylcholine receptor

Immunological analysis

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The interaction of the acetylcholine receptor (AChR) binding site domain with specific antibodies and with α-bungarotoxin (α-BTX) has been compared. The cloned and expressed ligand binding domain of the mouse AChR α-subunit binds α-BTX, whereas the mongoose-expressed domain is not recognized by α-BTX. On the other hand, both the mouse and mongoose domains bind to the site-specific monoclonal antibody 5.5. These results demonstrate that the structural requirements for binding of α-BTX and mcAb 5.5, both of which interact with the AChR binding site, are distinct from each other.

Acetylcholine receptor; α-Bungarotoxin; Ligand binding site; Monoclonal antibody 5.5

1. INTRODUCTION

The muscle nicotinic acetylcholine receptor (AChR) is a well characterized ligand-gated ion channel located in the postsynaptic folds of the neuromuscular junction. It is an integral membrane glycoprotein which forms an oligomeric complex composed of four subunits present in a molar stochiometry of $\alpha_2\beta\gamma\delta$ with an overall molecular weight of 250,000 Da [1,2]. Among these four subunits, the cholinergic binding site was shown to be located in a portion of the extacellular domain of the α-subunit, within a region containing the tandem cysteines at residues 192 and 193 [3–7]. Nevertheless, it should be noted that recent studies using specific affinity-labeling ligands suggest the involvement of additional amino acid residues from other domains of the α-subunit and possibly from other subunits in ligand binding [8].

As the natural ligand of the AChR, acetylcholine, has a low affinity to its receptor, the snake polypeptide neurotoxin, α-bungarotoxin (α-BTX), which has high affinity to the receptor and can be easily radiolabeled, has been particularly instrumental in the localization of the ligand binding site. However, not all the nicotinic AChRs bind snake neurotoxins [9,10] indicating that the binding sites for a α-BTX and for acetylcholine may be overlapping but not identical.

In an attempt to analyze this question, we have been studying the ligand binding region of muscle AChRs from animals such as certain snakes and the mongoose, which are known to be resistant to neurotoxins [10,13]. In order to understand the structural basis which confers this pharmacological specificity, we have cloned a fragment from the AChR α-subunit extracellular domain, comprising residues 122 to 205 from three different snake species [12,13], and from the mongoose [13]. We demonstrated that several major substitutions occur at the putative binding site of the snakes and mongoose, in the vicinity of cysteines 192 and 193. Expression of the cloned AChR fragments in bacteria and analysis of α-BTX binding on Western blots suggest that these substitutions may be sufficient to account form the special pharmacology of these receptors [13].

In this report we have employed immunological reagents for further analysis and characterization of the ligand binding site of the AChR. Both polyclonal antibodies directed against distinct domains of the AChR [5,14] as well as the monoclonal antibody 5.5.G.12 (mcAb5.5) [15] were used. Monoclonal antibody 5.5 was developed in our laboratory as a highly specific reagent directd against the AChR binding site and was shown to compete with cholinergic agonists and antagonists, including α-neurotoxins, in receptor binding, and to directly act as a cholinergic antagonist in blocking acetylcholine-induced sodium transport and modifying ionic channel properties [15–17]. We describe here the interaction of cloned and expressed ligand-binding domains from mouse and mongoose AChR, which vary in their response to α-BTX, with various antipeptide antibodies and with mcAb5.5. We demonstrate that the loss of α-BTX binding is not accompanied by a similar loss of specific antibody binding. Our results support

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the notion that, despite the physical vicinity or overlapping of their binding sites, there may be distinct requirements for the binding of acetylcholine and α-BTX.

2. MATERIALS AND METHODS

2.1. *Preparation of antibodies*

The recombinant α-subunit domains, coding for amino acids 122 to 205 from mouse and mongoose AChR, were subcloned into the expression vector pET-8c and expressed as described [13]. Polyclonal antibodies against these expressed fragments were raised in rabbits following injection of gel-purified bacterial expressed proteins as described [13]. Anti-p126–143 and p143–158 antibodies are directed against synthetic peptides corresponding to residues 126–143 and 143–158 of the *Torpedo* AChR α-subunit, respectively [5,14]. McAb5.5 is directed against the acetylcholine binding site of the AChR [15], and mcAb5.14 is directed against a highly immunogenic determinant located at a 14 kDa C-terminal fragment of the AChR α-subunit [18]. Ascitic fluids containing these monoclonal antibodies were employed [15].

2.2. *Western blot overlays*

Bacterially expressed α-subunit domains from mongoose and mouse AChR or partially purified *Torpedo* AChR were resolved by SDS-PAGE, and blotted onto nitrocellulose paper (Schleicher and Schuell). The blots were overlayed with polyclonal or monoclonal antibodies as described [5] and further probed with either iodinated protein A (for antipeptide antibodies) or iodinated goat-anti mouse IgG antibody (for monoclonal antibodies), or overlayed with ^{125}Iα-BTX [6].

2.3. *In vitro translation and immunoprecipitation*

The cloned α-subunit domains were subcloned into pBluescript or pSP65 and RNA was transcribed from linearized vectors using T3 or SP6 RNA plymerases, respectively. In-vitro translation was performed in a rabbit reticulocyte lysates (Promega). Proteins were labeled throughout translation with [^{35}S]methionine (Amersham). Prior to immunoprecipitation with polyclonal or monoclonal antibodies, the lysate was pre-cleared with sepharose-conjugated protein A or goat anti-mouse IgG, respectively. Immunoprocipitation of lysate proteins was performed as described [19]. The precipitated proteins were eluted from the beads, separated by SDS-PAGE on 15% gels, and visualized by fluorography.

3. RESULTS

The cloned α-subunit domains from mouse and mongoose AChR share 95% homology, with major substitutions in the vicinity of the putative ligand binding site. These domains are 82% homologous to the respective domain of the *Torpedo* AChR ([13]; Fig. 1, upper panel). We tested whether the cloned and expressed mouse and mongoose proteins can cross-react with various antibodies raised against components of the *Torpedo* AChR. To that end, lysates from bacteria expressing the cloned mouse and mongoose domains, as well as purified *Torpedo* AChR, were resolved in 15% acrylamide gels and blotted onto nitrocellulose. Identical blots were then overlayed with several polyclonal antibodies or with α-BTX, using *Torpedo* AChR as a positive control. As demonstrated in Fig. 1 (lower panel), polyclonal antibodies against the entire AChR (Fig. 1, lower panel, b), anti-p128–143 and p143–156

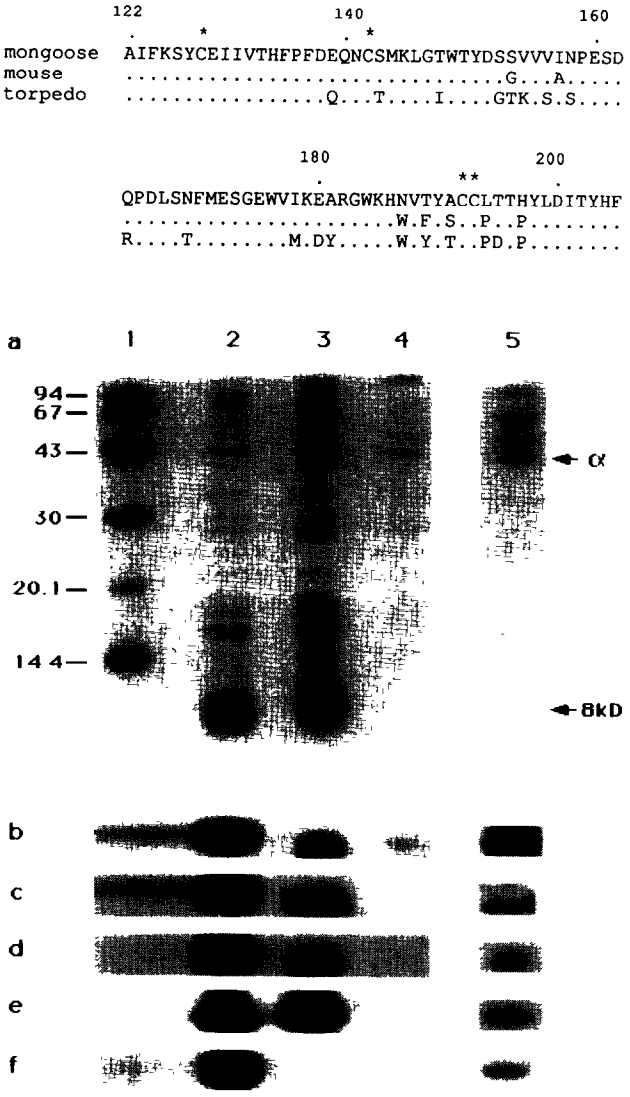

Fig. 1. Sequence and immunological profile of bacterially expressed AChR domains. (Upper panel) Comparison of the primary amino acid sequence of the mongoose, mouse and *Torpedo* AChR residues 122–205. The amino acids sequences are deduced from the cDNA sequence in the EMBL database and [13]. (Lower panel) Analysis of the bacterially expressed AChR domains with polyclonal antibodies. The mouse and mongoose domains, and purified *Torpedo* AChR, were resolved on 15% SDS-PAGE as described [13]. Identical samples were stained with Commassie brilliant blue (a) or transferred to nitrocellulose paper and interacted with polyclonal antibodies (b–e) or iodinated α-BTX (f). Immunoblots were further probed with ioidinated protein A. Lanes 1, molecular weight markers; 2 and 3, lysates from bacteria transfected with pET-8c containing the mouse or the mongoose 122–205 domain, respectively; 4, lysate from bacteria transfected with pET-8C; 5, *Torpedo* AChR. a, Coomassie brilliant blue staining; b, immunoblot with anti-*Torpedo* AChR antibodies; c and d, immunoblot with anti-p128–143 or anti-p143–158 antibodies, respectively; e, immunoblot with antibodies directed against the bacterially expressed mongoose domain; and f, overlay of a similar blot with [^{125}I]α-BTX. b–f present only the 8 kDa region in lanes 2–4, and the α-subunit (40 kDa) region in lanes 5.

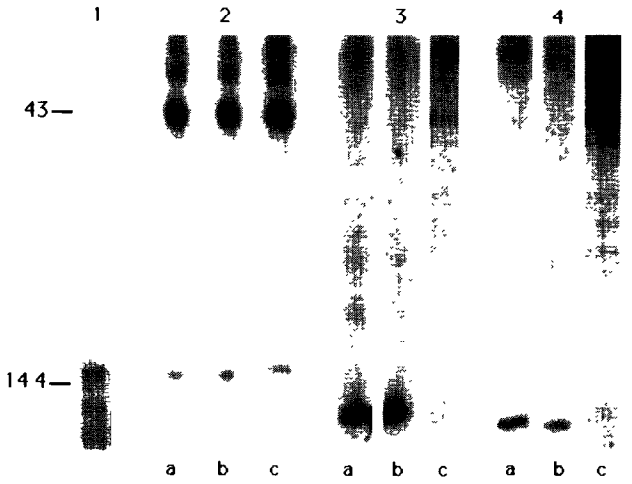

Fig. 2. Immunological profile of in vitro expressed AChR domains. Cell free expression was performed in rabbit reticulocytes as described in section 2. Lysates expressing an irrelevant control mRNA (panel 1), the entire mouse α-subunit (panel 2), the mouse domain coding for residues 122–205 (panel 3) and the mongoose domain coding for residues 122–205 (panel 4) were immunoprecipitated with the following polyclonal and monoclonal antibodies: anti-p143–158 (lanes a), mcAb5.5 (lanes b) and mcAb5.14 (lanes c). The immunoprecipitated proteins were resolved by 15% SDS-PAGE, and visualized by fluorography. The difference in band intensity results from the expression of a higher amount of labeled mouse domain in the initial in vitro translation reaction (data not shown), or probably due to the existence of an additional methionine in the mouse sequence (Fig. 1, upper panel).

(Fig. 1, lower panel, c and d, respectively) were able to recognize an 8 kDa protein in bacteria that express the α-subunit domains. As demonstrated by Coomassie brilliant blue staining, this 8 kDa protein constitutes the major component of the total proteins expressed in plasmid-harboring bacteria (Fig. 1, lower panel, a). The *Torpedo* AChR interacted with antibodies raised against gel-purified, expressed domain from the mongoose α-subunit (Fig. 1, lower panel, e), as well as with antibodies raised against a similar domain from the expressed mouse domain (data not shown). On the other hand, the expressed domains retained their unique pharmacological properties on the Western blot. Namely, as reported earlier [13], the mouse fragment and the entire *Torpedo* α-subunit interacted with iodinated α-BTX while the mongoose fragment was not recognized by the toxin (Fig. 1, lower panel, f).

As the bacterially expressed proteins are not soluble, we used cell-free expression in a eukaryotic system to determine whether the same immunological profile would be manifested also with soluble fragments. The mouse and mongoose fragments, as well as the entire mouse AChR subunit, were in vitro transcribed and translated using rabbit reticulocyte lysates. Under these conditions, the expressed domains are soluble and can therefore be analyzed by immunoprecipitation. As demonstrated in Fig. 2, panels a, the in vitro expressed mouse and mongoose fragments can interact with anti-peptide antibodies against the *Torpedo* receptor domain 143–158, as was demonstrated for the bacterially expressed fragments (Fig. 1). The in vitro expressed proteins could also be precipitated with anti-*Torpedo* AChR antibodies and with antibodies elicited against the bacterially expressed domains (data not shown). Thus, soluble receptor domains expressed in a eukaryotic system show the same cross-reactivity with various antibodies demonstrated by the denatured, immobilized, bacterially expressed receptor fragments.

Monoclonal antibodies were instrumental in the study of the structure–function relations of various domains within the AChR, such as the ligand binding site and the major immunogenic regions involved in the pathology of *Myasthania gravis* [15,18,20]. These reagents are directed against a single immunogenic determinant, and could be used to probe into the conformation of specific domains within the AChR. Monoclonal antibody 5.5 is especially interesting in this respect as this reagent is directed against the ligand binding site of the AChR in a conformation-dependent manner [15]. Thus it was interesting to determine whether this antibody would interact with the cloned α-subunit domains, expressed both in vitro and in bacteria. As a control we used mcAb5.14, a monoclonal antibody directed against a highly immunogenic domain in the cytoplasmic region of the α-subunit [18], which is not present in our cloned fragments.

As demonstrated in Figs. 2 and 3, the α-subunit domains were recognized by mcAb5.5 when expressed in a cell free system or in bacteria. McAb5.5 immunoprecipitated the in vitro expressed mouse and mongoose domains (Fig. 2, lanes b) as well as the entire α-subunit, while mcAb5.14 precipitated only the entire expressed mouse α-subunit (Fig. 2, lanes c). Similarly, bacterially expressed α-subunit domains immobilized on Western blots were identified by mcAb5.5 (Fig. 3, panel b) but not with mcAb5.14 (Fig. 3, panel c). Both antibodies reacted with Torpedo AChR α-subunit. (The bands observed at molecular weight higher than the α-subunit, probably represent dimers of α-subunit or heterdimers with the α-subunit.) These experiments demonstrate that the immunological behavior of the mouse and mongoose expresed domains is similar in both expression systems.

4. DISCUSSION

Our results demonstrate that two reagents, both of which are specifically directed against the AChR binding site, show different sequence specificity. α-BTX is widely used as an affinity ligand for muscle AChR α-subunit, while not recognizing other types of α-subunits such as certain subtypes present in the central nervous system [9,21]. In addition, α-BTX does not interact with the AChR α-subunit from various organisms that are resistant to snake toxins, such as certain snakes and the

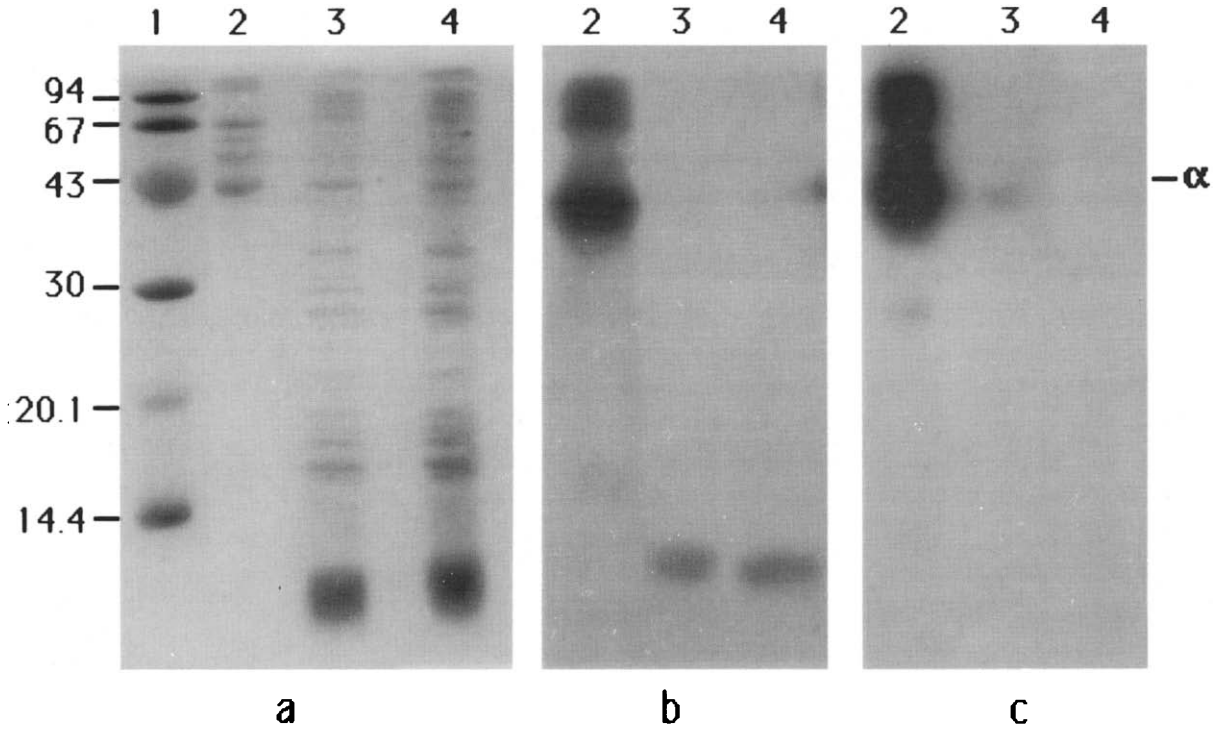

Fig. 3. Interaction of bacterially expressed AChR domains with monoclonal antibodies. Lysates from bacteria expressing the mouse and mongoose AChR domains, as well as *Torpedo* electric organ AChR, were resolved by SDS-PAGE and immobilized on nitrocellulose as described in the legend to Fig. 1, lower panel. Idential gels were stained with Coomassic brilliant blue (panel a), or blotted and overlayed with mcAb5.5 (panel b) or mcAb5.14 (panel c). Immunoblots were further probed with iodinated goat anti-mouse IgG antibodies. Lanes 1, molecular weight markers; 2, *Torpedo* AChR; 3, expressed mouse domain; 4, expressed mongoose domain. The bands observed at molecular weight higher than the α-subunit, probably represent dimers of α-subunit or heterodomers with the α-subunit.

mongoose [12,13]. Our study using the bacterially expressed proteins demonstrates that the specificity of this interaction is directly determined by the primary amino acid sequence (Fig. 1). This conclusion is supported by the findings of other groups, using synthetic peptides [22] or fusion proteins [23].

Monoclonal antibody mcAb5.5 is directed against the cholinergic binding site, as could be deduced by the following criteria: competition with α-BTX for binding the entire *Torpedo* AChR [15] as well as for binding to proteolytic fragments harboring the putative cholinergic binding site [7,18]; inhibition of antibody binding to the AChR by nicotinic agonists, [15]; and antibody-mediated competitive inhibition of ligand-induced ion channels in cells expressing the muscle nicotinic AChR [16,17]. This antibody is conformation-specific, as it does not recognize denatured AChR [15]. Our results demonstrate that this antibody can recognize the cloned α-subunit domains, expressed both in a soluble form in vitro and in an insoluble form in bacteria. The interaction of mcAb5.5 with the immobilized proteins, that were subjected to electrophoresis in denaturing conditions, suggests that these domains can undergo partial renaturation upon transfer, which is sufficient to restore the structure of the antibody binding site.

The different interaction of α-BTX and mcAb5.5 with the mouse and mongoose domains suggests that these two reagents recognize different moieties within the ligand binding site. The cholinergic binding site is designed to be recognized by acetylcholine, a small molecule, and it is plausible to assume that selective pressure against toxing binding would design a sequence that is able to recognize acetylcholine but not α-BTX. Our results demonstrate that the amino acid substitutions involved in conferring toxin resistance did not abolish the interaction of the α-subunit domain with mcAb5.5. Thus, mcAb5.5 is directed against an overlapping, but not identical, structure within the cholinergic binding site. These results suggest that the specific requirements for binding of various reagents such as α-BTX, mcAb5.5, and acetylcholine, all of which interact with the AChR binding site, are distinct form each other. Determination of the specific residues required for mcAb5.5 recognition, and comparison with the known specificity of acetylcholine binding site, could provide further insight into this question.

An obvious advantage of a cholinergic reagent that is not limited by the receptor specificity for α-BTX, is the ability to use such a reagent in the study of a subgroup of nicotinic AChRs which do not bind α-BTX. Even in animals that harbor α-BTX-sensitive muscle AChRs, the AChRs from other tissues, including the

central nervous system, differ in their capability to bind α-BTX. As demonstrated in this paper, mcAb5.5 is a reagent that can bind to the ligand binding site also of AChRs that do not recognize α-BTX. Thus, mcAb5.5 may be used as an affinity reagent in the purification, localization and characterization of ligand binding sites in such receptors.

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