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# Affinity labelling of neuronal acetylcholine receptors localizes acetylcholine-binding sites to their β-subunits

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Neuronal nicotinic acetylcholine receptors (AChRs) from brains of chickens and rats consist of two types of subunits, α and β, of which α shares some antigenic determinants with α-subunits from AChRs of electric organ and muscle [(1986) Biochemistry 25, 2082–2093; (1986) J. Neurosci. G, 3061–3069; (1986) Proc. Natl. Acad. Sci. USA, in press]. Here we demonstrate that after reduction with dithiothreitol (DTT) the AChRs can be specifically labelled with the acetylcholine-binding site directed reagent 4-(*N*-maleimido)benzyltri [$^3$H]methylammonium iodide. Labelling of the β-subunits of neuronal nicotinic AChRs indicates that the acetylcholine-binding site, and amino acids which may be homologous to Cys 192–193 of the α-subunits of AChRs from electric organ and muscle, are located on the β-subunit of neuronal AChRs. These results suggest that although neuronal nicotinic AChRs have some structural homologies to AChRs from muscle and electric organs, the AChRs from these sources are quite distant relatives in an extended gene family.

Acetylcholine receptor; Nicotine; Affinity labeling; Monoclonal antibody; (Brain)

## 1. INTRODUCTION

Avian and mammalian brains contain both nicotinic binding sites which have high affinity for nicotine and acetylcholine but not for α-bungarotoxin (αBgt), and distinct αBgt-binding sites [2–5]. The function of the αBgt-binding sites remains obscure, since there have been numerous reports that αBgt fails to block neuronal AChR function [6–8]. Neuronal nicotinic AChRs which do not bind αBgt have remained elusive until very recently, due to the lack of a suitable biochemical probe. We have used monoclonal antibodies (mAbs) to purify and characterize these neuronal AChRs. mAb 35, raised to AChRs from *Electrophorus* electric organ, crossreacted with an AChR from chicken brain [9]. This AChR bound nicotine with high affinity, but did not bind αBgt [1,2]. The immunoaffinity-purified brain AChR had two subunits, of $M_r$ 49000 (α) and 59000 (β), the smaller of which bound antisera and mAbs to α-subunits from AChRs of electric organ and muscle [1]. Antisera raised to the chicken brain AChR specifically blocked the acetylcholine-induced depolarization of chick ciliary ganglion neurons in culture [10], demonstrating that the component bound by mAb 35 was a functional AChR. mAbs raised to the chicken brain AChR identified a second chicken brain AChR subtype which bound mAb 35 only weakly, and consisted of apparently the same α-subunit but a different β-subunit, β′, of $M_r$ 75000 (submitted). mAb 270, raised to chicken brain AChRs, crossreacted with a homologous AChR from rat brain, and was used to immunoaffinity purify this receptor. The receptor from rat brain is homologous to the Aβ′ subtype from chicken brain and consists of two types of subunits, α ($M_r$ 51000) and β′ ($M_r$ 79000). mAb 270 bound to the α-subunits of AChRs from the brains of chickens and rats, as did antisera to α-subunits of *Torpedo* AChR [3].

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The localization of the neurotransmitter-binding sites to the $\alpha$-subunits of electric organ and muscle AChRs was determined using the affinity alkylating reagents bromoacetylcholine (BAC) and 4-(*N*-maleimido)benzyltri[$^3$H]methylammonium iodide (MBTA) [11–13]. Reaction of these reagents depends upon the fact that there is a disulfide bond which is easily reduced directly adjacent to the acetylcholine-binding site. In *Torpedo* AChR, this disulfide bond is believed to be formed between Cys 192–193 of the $\alpha$-subunit [14]. We have previously demonstrated that reduction followed by affinity alkylation of brain AChRs with BAC inhibits [$^3$H]nicotine binding to these receptors [2], suggesting that residues homologous to Cys 192–193 are conserved in neuronal AChRs. Here, we show that MBTA similarly blocks [$^3$H]nicotine binding and that [$^3$H]MBTA specifically labels the $\beta$-subunit of AChRs from brains of chickens and rats.

## 2. MATERIALS AND METHODS

### 2.1. *Antibodies*

mAb 35 was raised to AChRs from *Electrophorus* electric organ [15]. mAbs 270 and 285 were prepared from rats immunized with affinity-purified chicken brain AChR. Their production and characterization will be described elsewhere (Whiting et al., submitted). Preparation of antibody affinity columns has been described [1,3]. mAb 35 was coupled to CNBr-activated Sepharose CL4B at 6 mg protein/ml, mAb 270 at 6 mg protein/ml, and mAb 285 at 9.6 mg protein/ml.

### 2.2. *Brain extracts*

Rat and chicken brains were obtained from Pel-Freeze Biologicals. Triton X-100 extracts of brains were prepared as in [1,3].

### 2.3. *[$^3$H]Nicotine-binding assay*

[$^3$H]Nicotine (DL-[*N-methyl*-$^3$H]nicotine; spec. act. 68.8 Ci/mmol, New England Nuclear) binding to AChR immobilized on mAbs was determined as described [2].

### 2.4. *Labelling of AChRs with [$^3$H]MBTA*

Detergent extracts of 70–90 chicken brains (250–350 ml, 0.2–0.3 nM [$^3$H]nicotine-binding sites) or 30–40 rat brains (160–190 ml, 0.3–0.5 nM [$^3$H]nicotine-binding sites) were recirculated for 15 h at 4°C through 200 $\mu$l mAb 35 Sepharose and mAb 285 Sepharose (chicken brain extract) or mAb 270 Sepharose (rat brain extract). mAb 35 Sepharose (200 $\mu$l) was also gently shaken for 15 h at 4°C with 50 pmol affinity-purified *Torpedo* AChR, in 500 $\mu$l final volume 10 mM Na phosphate, pH 7.5, 100 mM NaCl (PBS) containing 0.5% Triton X-100. The affinity gels were divided into two aliquots in 1.5 ml microfuge tubes, washed with 4 × 1 ml PBS, 0.5% Triton X-100 by pelletting and resuspension, and then reduced for 40 min with 200 $\mu$l of 1 mM DTT in the same buffer. A *Torpedo* AChR control tube was reduced in the presence of 1 $\mu$M $\alpha$Bgt. The aliquots were then rapidly washed with 1 ml PBS, 0.5% Triton X-100 and then alkylated for 3 min with $1 \times 10^{-5}$ M [$^3$H]MBTA (spec. act. 1900 cpm/pmol, a gift from Dr Mark McNamee) in the presence or absence of 1 $\mu$M $\alpha$Bgt (for *Torpedo* AChR) or 2 mM nicotine (for brain AChRs) in 200 $\mu$l final volume. The aliquots were rapidly washed with 4 × 1 ml PBS containing 0.5% Triton X-100. Bound protein was then eluted from the antibody columns by 5 min incubations with 2 × 150 $\mu$l volumes of 50 mM sodium citrate, pH 3.0, 0.1% Triton X-100, and rapidly neutralized with 1 M Tris. The eluates were shaken for 2 h at 4°C with 50 $\mu$l goat anti-rat IgG coupled to Sepharose 4B (to absorb any IgG heavy and light chains which may have been nonspecifically labelled with the [$^3$H]MBTA and subsequently leaked from the column during the elution procedure). The eluate was then lyophilized, resuspended in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and resolved by SDS 10% PAGE, as described [1]. The acrylamide gels were then impregnated with Enhance (New England Nuclear), dried, and fluorographed for 4–21 dys at −70°C using preflashed Kodak XAR film.

## 3. RESULTS

Affinity labelling of brain AChRs was investigated by immuno-isolating the AChRs on mAbs coupled to Sepharose. mAb 35 was used to immobilize the chicken brain AChR subtype $\alpha\beta$, to which it preferentially binds; mAb 35 also binds to the $\alpha\beta'$ AChR subtype, but with lower affinity (submitted). mAb 285 was used to immobilize the

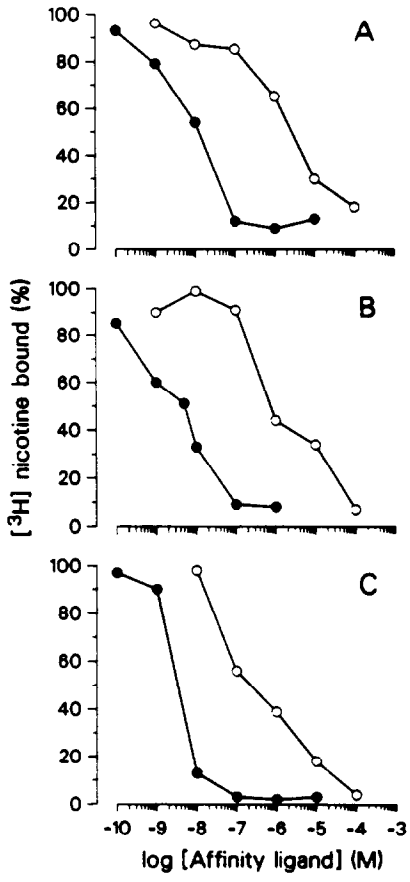



Fig.1. Affinity labelling with BAC (●) and MBTA (○) of chicken brain AChR immobilized upon (A) mAb 35, and (B) mAb 285, and (C) rat brain AChR immobilized upon mAb 270. Chicken brain detergent extracts (600 μl) (A,B) or 200 μl rat brain detergent extract (C) were gently shaken in 1.5 ml microfuge tubes for 15 h at 4°C with 30 μl goat anti-rat IgG Sepharose and (A) 0.5 μl mAb 35 (anti-*Torpedo* AChR titer 49 μM αBgt-binding sites), (B) 0.5 μl mAb 285 (anti-chicken brain AChR titer 1.4 μM nicotine-binding sites), or (C) 1.0 μl mAb 270 (anti-chicken brain AChR titer 0.33 μM nicotine-binding sites). The aliquots were washed by brief pelletting and resuspension with 1 ml PBS, 0.5% Triton X-100, and then reduced for 40 min at room temperature with 1 mM DTT in the same buffer. After washing with 1 ml PBS, 0.5% Triton (without DTT) the aliquots were resuspended in 100 μl of various concentrations of BAC (prepared by the method of Chiou and Sastry [26]), or MBTA [11] in the same buffer. After 5 min at room temperature, the aliquots were washed with 4 × 1 ml PBS, 0.5% Triton X-100 to remove noncovalently bound affinity ligand and then incubated for 10 min in 0.1 mM dithiobis(2-nitrobenzoic acid). After washing with 2 × 1 ml PBS, 0.5% Triton, the aliquots were incubated for 15 min at room temperature, in 50 μl [$^3$H]nicotine (20 nM) in PBS, 0.5% Triton X-100. The aliquots were rapidly washed at 4°C with 3 × 1 ml ice-cold PBS, 0.5% Triton X-100. Bound protein and [$^3$H]nicotine were eluted by brief incubation with 100 μl of 2.5% SDS, 5% mercaptoethanol and radioactivity determined by taking the eluate for scintillation counting [2]. A parallel incubation, in which the affinity alkylation step was omitted, was carried out, and the [$^3$H]nicotine binding considered as 100% and all other values expressed relative to this. Each point is the mean of triplicate aliquots. Nonspecific binding of [$^3$H]nicotine was determined by incubation in the absence of mAb. In all experiments it represented less than 5% of the total binding, and has been subtracted from all values.

chicken brain AChR $\alpha\beta'$ subtype. This mAb is directed to the $\beta'$-subunit, and thus specifically binds the $\alpha\beta'$ AChR subtype (submitted). mAb 270, which is directed to the $\alpha$-subunit [2,3], was used to immobilize AChRs from rat brain.

Fig.1 shows the affinity alkylation of AChRs from brains of chickens and rats by both BAC and MBTA, as measured in terms of inhibition of [$^3$H]nicotine binding to immuno-isolated AChRs. This confirms our previous data showing affinity labelling with BAC of chicken brain AChRs immobilized upon mAb 35 Sepharose [1,2]. For both receptor subtypes from chicken brain (fig.1A,B) and receptor from rat brain (fig.1C), the affinity labels were able to inhibit greater than 90% of the [$^3$H]nicotine binding. Higher concentrations of MBTA than BAC were required to affinity label the brain AChRs. Since MBTA is an antagonist and BAC an agonist, this observation is consistent with the demonstration that these receptors have high-affinity binding for nicotinic cholinergic agonists and considerably lower affinity binding for nicotinic cholinergic antagonists [2]. In contrast, higher concentrations of BAC than MBTA are required to affinity label the ganglionic type neuronal AChRs of PC12 cells [16], which is consistent with their considerably lower affinity for nicotinic agonists compared to AChRs from brain [17].

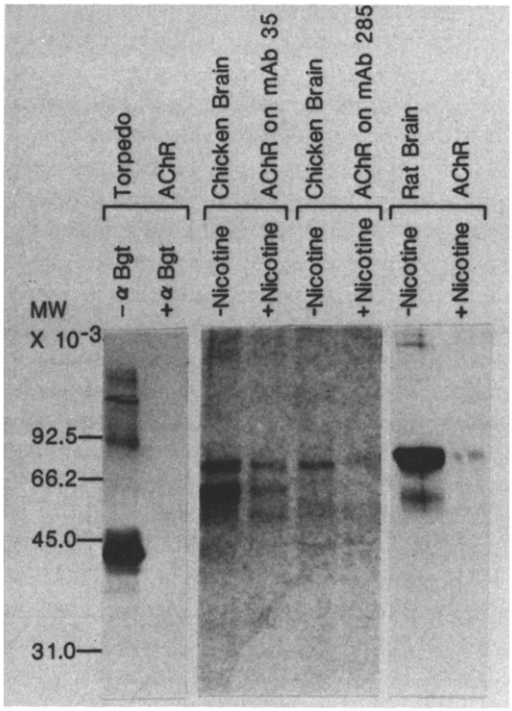



Fig.2. Fluorogram of SDS-PAGE of AChRs from *Torpedo* electric organ, chicken brain, and rat brain labelled with [$^3$H]MBTA. Labelling procedures and SDS-PAGE were carried out as described in section 2. Apparent $M_r$ values were determined by resolving $M_r$ standards (BioRad) on the same gel and staining for protein with Coomassie blue.

The AChR subunit labelled by the affinity ligand was determined by immobilizing AChR on mAb Sepharose, alkylating with [$^3$H]MBTA in the presence or absence of excess cholinergic ligand, eluting the labelled protein, and subsequently analyzing by SDS-PAGE and fluorography. The feasibility of this approach was shown by demonstrating that *Torpedo* AChR, immobilized on mAb 35 Sepharose, could be specifically labelled upon its $\alpha$-subunit (fig.2). When AChR from chicken brain was immobilized on mAb 35 Sepharose and affinity labelled with [$^3$H]MBTA, two polypeptides were labelled, $M_r$ 60600 ± 1700 and $M_r$ 75600 ± 400 (mean ± SD, three labelling experiments), which correspond to the $\beta$-subunit ($M_r$ 59000 [1]) and $\beta'$-subunit ($M_r$ 75000, submitted) of the chicken brain AChR subtypes (fig.2). The labelling was specific, since it was inhibited by 2 mM nicotine (fig.2). The $\beta$-subunit was always more intensely labelled than the $\beta'$-subunit, consistent with the higher affinity of mAb 35 for the $\alpha\beta$ AChR subtype than the $\alpha\beta'$ subtype. When mAb 285 Sepharose was used to immobilize specifically the chicken brain AChR $\alpha\beta'$ subtype, a single polypeptide was labelled, $M_r$ 75400 ± 400 (mean ± SD, two labelling experiments), corresponding to the $\beta'$-subunit of the AChR $\alpha\beta'$ subtype (fig.2). Similarly, rat brain AChR, immobilized on mAb 270 Sepharose, was specifically labelled with [$^3$H]MBTA on a single polypeptide, $M_r$ 79600 ± 500 (mean ± SD, three labelling experiments) corresponding to the $\beta'$-subunit of the rat brain AChR ($M_r$ 79000, [3]). For both chicken brain AChR subtypes and the rat brain AChR, the [$^3$H]MBTA labelling could also be blocked by 1 mM carbachol (not shown), confirming that the [$^3$H]MBTA was specifically labelling the neurotransmitter-binding site of the neuronal AChRs.

## 4. DISCUSSION

The affinity labelling of AChRs after reduction with DTT suggests that amino acid residues which may be homologous to Cys $\alpha$192–193 are conserved in AChRs from brain. The $\alpha$Bgt-binding proteins from brains of chicks [18] and rats [19] can also be affinity labelled with BAC and MBTA. Similarly, it has been shown that the functional AChRs on both rat PC12 cells [16] and chick ciliary ganglion neurons [10] can be affinity labelled. These ganglionic-type AChRs share some antigenic determinants with AChRs from brain [10,20] but are pharmacologically distinct, having several orders of magnitude lower affinities for cholinergic agonists [17,21]. Thus, amino acid residues homologous to Cys $\alpha$192–193 of electric organ and muscle AChRs may have been conserved in the members of what appears to be an extended gene family consisting of muscle and electric organ AChRs, brain AChRs, ganglionic AChRs, and brain $\alpha$Bgt-binding proteins.

The observation that the $\beta$-subunits of the AChRs from chicken and rat brains were labelled by [$^3$H]MBTA was surprising because we have previously observed that it was the smaller $\alpha$-subunit of these receptors which shared antigenic determinants with $\alpha$-subunits from AChRs of elec-

tric organ and muscle, and thus was considered to be the $\alpha$-subunit homologue [1,3]. In particular, the main immunogenic region was conserved on the $\alpha$-subunit of AChRs from chicken brain [1], although not on AChRs from rat brain [2,3]. The main immunogenic region is a domain on the extracellular surface of $\alpha$-subunits of AChRs from electric organ and skeletal muscle against which the majority of antibodies are directed in an immune response to native AChRs [22]. Using mAbs, it has been localized on $\alpha$-subunit from *Torpedo* to somewhere between amino acid residues 46 and 127 [23]. The four kinds of subunits of AChRs from electric organs and muscle are thought to have evolved from a single primordial subunit by repeated gene duplication [24,25]. The subunits of neuronal nicotinic AChRs and $\alpha$Bgt-binding components probably also evolved from this primordial receptor along different lineages of this extended gene family. If it is assumed that the primordial subunit had homologues of both the main immunogenic region and $\alpha$192–193, it appears that in the course of gene duplication and subsequent evolution both of these features continued to be expressed in $\alpha$-subunits of AChRs from muscle, while in the case of neuronal AChRs the antigenic phenotype persisted in $\alpha$-subunits while the Cys $\alpha$192–193 analogues persisted in $\beta$-subunits.

The two chicken brain AChR subtypes, $\alpha\beta$ and $\alpha\beta'$, have virtually identical affinity for L-nicotine and similar or identical $\alpha$-subunits, but are distinguishable by their $\beta$-subunits, which have different apparent molecular masses and bind different mAbs (submitted). Thus, localizing the cholinergic binding site to the $\beta$-subunits of these AChRs was unexpected. The domain containing the neurotransmitter-binding site must be highly conserved on both of these $\beta$-subunits.

It is clear that although certain key structures, such as homologues of Cys $\alpha$192–193, are conserved in some subunits of all AChRs, the AChRs from neurons have diverged considerably from those in electric organ and skeletal muscle. The application of molecular genetic techniques will allow determination of the amino acid sequences of the neuronal AChR subunits and will eventually lead to a better understanding of the structure and evolution of these neuronal AChRs and their function in neurotransmission.

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