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Neuronal nicotinic acetylcholine receptor β-subunit is coded for by the cDNA clone α_4

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Acetylcholine receptors (AChRs) with high affinity for nicotine but no affinity for α-bungarotoxin, which have been purified from rat and chicken brains by immuno-affinity chromatography, consist of two types of subunits, α and β [1,2]. The β-subunits form the ACh binding sites [3]. Putative nicotinic AChR subunit cDNAs α_3 and α_4 have been identified by screening cDNA libraries prepared from rat PC12 cells and rat brain with cDNA probes encoding the mouse muscle AChR α-subunit. Here we determine the amino-terminal amino acid sequence of the rat brain AChR β-subunit by protein microsequencing to be the same as amino acid residues 27–43 of the protein which could be coded by α_4. Further, we present evidence consistent with a subunit stoichiometry of $\alpha_3\beta_2$ for this neuronal nicotinic AChR.

Neuronal acetylcholine receptor; Nicotine; cDNA; Gas phase protein microsequencing

1. INTRODUCTION

In both the avian and mammalian brain there are nicotinic AChRs which have high affinity for nicotine and ACh, but not for α-bungarotoxin (αBgt) (review [6]). Additionally, there are distinct αBgt-binding sites found in the nervous system, the nature of which remains obscure since there have been many reports that αBgt fails to block neuronal AChR function (review [7]). In chick ciliary ganglion cells and rat PC12 cells, there are nicotinic AChRs which, like those in brain, do not bind αBgt [8–10]. However, these AChRs differ from those found in the brain, in having a considerably lower affinity for nicotine and ACh [11–13]. Thus, pharmacological and physiological evidence suggest the existence of a family of neuronal nicotinic AChRs.

We have used monoclonal antibodies (mAbs) as tools to purify and characterize AChRs from brains of chickens and rats [1–3,14]. These AChRs exhibit high affinity for nicotine and ACh, but do not bind αBgt [14]. Structurally they consist of two kinds of subunits, α and β. In chicken brain, two AChR subtypes have been identified which apparently have the same α-subunits (M_r 49000) but different β-subunits (β M_r 59000 and β′ M_r 75000) ([3]; Whiting, P. et al., submitted). AChRs from rat brain are composed of α-subunits (M_r 51000) and β-subunits (M_r 79000) [2]. These β-subunits are similar in molecular mass and immunologically homologous to chicken β′-subunits ([2]; Whiting, P. et al., submitted). It is the β-subunits of these AChRs which contain the ACh-binding site, as shown by affinity labeling with MBTA (4-(*N*-maleimido)benzyltrimethylammonium) after reduction with dithiothreitol [3]. This suggests that β contains cysteines analogous to α192–193 of α-subunits of AChR from *Torpedo* electric organ, because these are the residues of *Torpedo* α-subunits labeled by MBTA [15]. mAb 270, prepared against AChRs from chicken brain and specific for its α-subunits, has been utilized to histochemically localize AChRs in rat brain [16]

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and is a specific probe for the functional AChR of PC12 cells [13].

An alternative approach to identifying neuronal nicotinic AChRs uses molecular genetic techniques. By low stringency screening of a cDNA library prepared from PC12 cells with a probe prepared from mouse muscle AChR α-subunit cDNA, a putative neuronal AChR α-subunit clone, α_3, was isolated [4]. This cDNA clone hybridizes to areas of brain known to have high-affinity nicotine-binding sites, but also to areas known to have αBgt-binding sites [17]. Additionally, the PC12 mRNA species which hybridizes to α_3, like the αBgt-binding component of these cells, is not induced by culture of cells in β-nerve growth factor, whereas the functional AChR labeled by mAb 270 is induced greater than 4-fold by nerve growth factor, which suggests that α_3 may not code for a subunit of the PC12 AChR [13]. Recently, by low stringency screening of a cDNA library from rat hippocampus and hypothalamus, Goldman et al. [5] have reported the isolation of a second putative neuronal AChR α-subunit cDNA clone, α_4 [5]. This cDNA was reported to hybridize with rat brain in a pattern different from α_3 [5] and in some respects more like the pattern of labeling by mAb 270 [16]. From the deduced amino acid sequence it was not possible to determine the amino terminus of the processed protein, to determine what other subunits might be associated with the coded protein, or to show that it was a functional nicotinic AChR.

Here we report the amino acid sequence of the amino terminus of the β-subunit of the AChR which was immunoaffinity purified from rat brain using the α-subunit specific mAb 270. This sequence is the same as residues 27–43 of the cDNA clone α_4, indicating that the cDNA α_4 codes for the β-subunit of this neuronal AChR. Additionally, data is presented consistent with the idea that the subunit stoichiometry of the AChR of rat brain is $\alpha_3\beta_2$.

2. MATERIALS AND METHODS

AChR from rat brain was purified through two rounds of affinity chromatography upon mAb 270 coupled to Sepharose CL4B, as has been described [2]. AChR subunits were dissociated by SDS and resolved by electrophoresis in a 10% polyacrylamide gel (SDS-PAGE) [2]. The subunits were electroblotted at 25 V for 5 h onto a quaternary ammonium derivatized glass fiber sheet [18]. The protein bands were located by fluorescent staining [18], excised, and subjected to gas phase microsequencing [19]. The phenylthiohydantoin (PTH) amino acid derivatives from the Applied Biosystems model 470A protein sequencer were automatically analyzed on an Applied Biosystems model 120A PTH analyzer.

3. RESULTS AND DISCUSSION

SDS-PAGE of approx. 60 pmol of rat brain AChR with subsequent electroblotting onto derivatized glass fiber paper [18] and microsequence analysis of the β-subunit yielded the amino acid sequence data shown in fig.1. The low initial yield of the Edman degradation in fig.1 may be accounted for by the low efficiency of the electroblot procedure (quantitated using ^{125}I labelled proteins to be 42–65% efficient in our hands) and other losses that are inherent in the manipulation of such small quantities of protein. The indicated amino acid assignments were made with high confidence, and are identical to the deduced amino acid sequence (residue numbers 28–43) of the cDNA clone α_4 reported by Goldman et al. [5] (fig.2). Additionally, six other residues were assigned with less confidence, one of which was identical to residue 27, and four of which subsequently were found to be identical to residues 44–48 of the α_4 cDNA clone (not shown). The alignment of the amino-terminal amino acid sequence with the deduced sequence from α_4 indicates that the amino terminus of α_4 is five residues longer than both α_1 (muscle AChR) and the other putative neuronal AChR clone, α_3. The mature protein coded for by clone α_4 would be 601 amino acids in length, a size which corresponds reasonably well to the β-subunit of the rat brain AChR we previously purified (apparent M_r 76000 after endoglycosidase treatment) [2]. Also, clone α_4 contains cysteine residues at positions 192 and 193, which in electric organ and muscle AChR are known to be at or near the ACh-binding site, are disulfide linked and labeled by the affinity ligand MBTA after reduction with dithiothreitol [15]. This is in agreement with our previous demonstration that the ACh-binding site of the rat brain AChR also has a

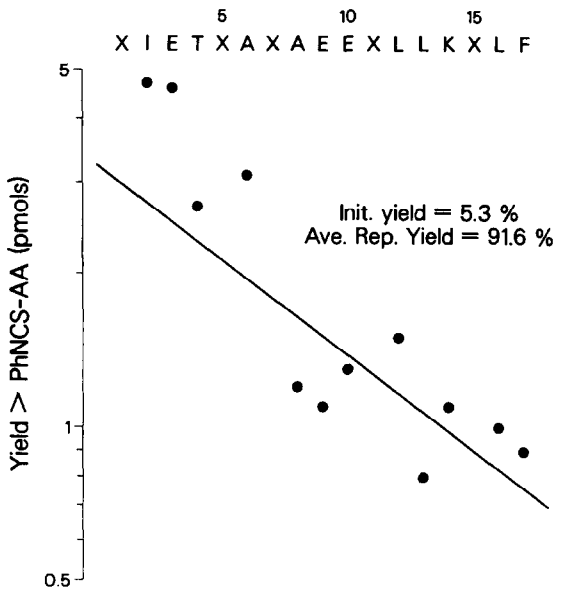

Fig.1. Amino-terminal amino acid sequence of β-subunit of rat brain AChR.

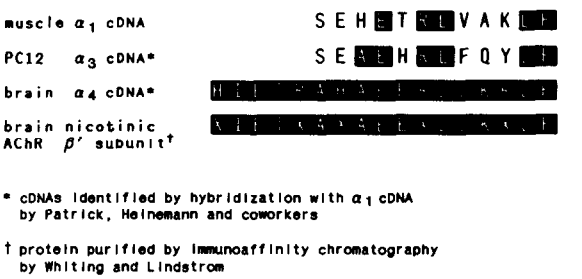

Fig.2. Alignment of deduced amino acid sequences for AChR α-subunits with the amino-terminal amino acid sequence of the β-subunit of AChR from rat brain.

reducible disulfide bond nearby, and is located in the β-subunit of this AChR [3].

The areas of brain expressing RNA homologous to clone α_4, as determined by in situ hybridization [5], appear to be similar to the pattern observed when thin sections of rat brain are labeled with mAb 270 [16]. However, α_4 was not reported to hybridize to superior colliculus [5], which is strongly labeled by mAb 270 [16]. There are two likely explanations. First, we showed that the sites in superior colliculus are located on endings of retinal ganglion cells [16], and mRNA would be expected to be located in the ganglion cell bodies rather than at this presynaptic location. Second, in both chick (Whiting et al., submitted) and rat brain (unpublished) there are subtypes of AChR which differ in their β-subunits and histological location. Thus, the subtype coded by α_4 may not be expressed in ganglion cells.

We also obtained the amino-terminal amino acid sequence of the 51 kDa α-subunit of AChR from rat brain (not shown). This sequence data, together with other biochemical data, has been used to investigate the subunit stoichiometry of the neuronal AChR macromolecule (table 1). We know from binding of mAb 270 that there are at least two α-subunits in each AChR molecule. AChRs immobilized on mAb 270 coupled to Sepharose can still bind ^{125}I-mAb 270 (unpublished). In the case of AChRs from chicken brain, we know from similar mAb-binding experiments that there must be at least two α-subunits [2] and two β-subunits in each AChR (Whiting et al., submitted). Thus, we are confident that the subunit stoichiometry is $\alpha_n \geqslant 2\beta_n \geqslant 2$. The data in table 1 is most consistent with $\alpha_3\beta_2$, which preserves the pentagonal symmetry with two ACh-binding subunits and three structural subunits of the $\alpha_2\beta\gamma\delta$-subunit structure of AChRs from *Torpedo* [20]. However the possibility of stoichiometries of $\alpha_2\beta_2$ or $\alpha_2\beta_3$ cannot be eliminated at this time.

Convergence of data derived from the biochemical approach and the molecular genetic approach to the characterization of neuronal nicotinic AChRs results in some conflict over nomenclature. All known AChR subunits share some sequence homology and therefore are thought to have evolved from a single primordial subunit [22,23]. The subunits of electric organ AChRs were initially referred to by molecular masses, but this led to confusion depending on exact calibration of molecular mass standards (e.g. [21]). They were then referred to as α, β, γ, and δ in order of increasing molecular mass. Several lines of evidence showed that α-subunits contained the ACh-binding site, primarily affinity labeling, which localized it to the proximity of cysteines α192–193 [15]. We referred to the smaller subunit of the AChRs which we have purified from brain [1,2] as 'α' because they were the smaller subunit and because they were bound by polyclonal sera to α-subunits from *Torpedo* AChR. The larger subunits which did not bind antisera to α-subunits

Table 1

Subunit stoichiometry of the neuronal nicotinic AChR from rat brain

Possible stoichiometry	Predicted			Observed		
	Apparent molecular mass[a]	α/β per cycle of sequenator[b]	α/β from SDS-PAGE	Apparent molecular mass[c]	α/β per cycle of sequenator[d]	α/β from SDS-PAGE[e]
$\alpha_2\beta_2$	260000	1	1			
$\alpha_3\beta_2$	310000	1.5	1.5	338000	1.39 ± 0.44	1.29 ± 0.18
$\alpha_2\beta_3$	339000	0.67	0.67			

[a] Apparent molecular mass was calculated from apparent molecular masses of subunits determined by SDS-PAGE (α M_r 51000 and β M_r 79000)

[b] Ratio of mol of α-subunit amino acid to β-subunit amino acid at each cycle of sequencing

[c] The apparent molecular mass of the AChR was determined from sucrose gradient analysis using ^{125}IαBgt labelled *Torpedo* AChR monomers and dimers as standards [2]. The $S_{20,w}$ value for brain AChR was found to be 10.6, which is equivalent to an apparent molecular mass of 338 kDa, calculated using the method of Martin and Ames [24]

[d] The N-terminal amino acid sequence of the α- and β-subunits were determined as described in section 2. The mole ratio (mean ± standard deviation) of α/β subunit is shown for the first eleven sequencing cycles

[e] Ratio α/β of autoradiogram band intensities of radioiodinated rat brain AChR resolved by SDS-PAGE [2]. The value shown is the mean ± standard deviation derived from four analyses. This assumes that ^{125}I is incorporated at an equal weight ratio in each subunit

from *Torpedo* were referred to as 'β'. We subsequently showed that β was the ACh-binding subunit because it could be affinity labeled by reagents specific for cysteines 192, 193 of α-subunit of AChR from *Torpedo* electric organ [3]. Patrick and coworkers [4,5] referred to the cDNA clones they isolated as 'α' because they had significant sequence homology to subunits from muscle AChR, most importantly, to cysteine residues at positions 192 and 193, which are located in the α but not the β, γ, or δ subunits of AChRs from electric organs and muscle.

Some standardization of nomenclature for AChR subunits may now be required. For instance, what we refer to as rat brain AChR β-subunit [2], and what Goldman et al. [5] refer to as α_4, we have shown to be the ACh-binding subunit [3]. It might be clearest to refer to it as the 'ACh-binding subunit'. The other subunit of neuronal AChRs has antigenic determinants in common with electric organ AChR α, may or may not contain cysteines 192, 193; but is not affinity labeled by MBTA, and like β, γ, and δ subunits, probably does not significantly bind ACh. It could be referred to as a 'structural subunit'.

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