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β3 subunit is present in different nicotinic receptor subtypes in chick retina

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Abstract

Although the neuronal nicotinic β3 subunit was cloned several years ago, it has only recently been shown to form heteromeric channels when associated with other nicotinic subunits, and very little information is available concerning its assembly in the native nicotinic receptors of the nervous system. Using subunit-specific antibodies and immunoprecipitation experiments, we have identified the retina as being the chick central nervous system (CNS) area that expresses the highest level of the β3 subunit. Sequential immunopurification experiments showed that there are at least two populations of β3-containing receptors in chick retina: in one, the β3 subunit is associated with the α6 and β4 subunits; in the other more heterogeneous population, the β3 subunit is associated with the α2, α3, α4, β2 and β4 subunits. Both of these receptor populations bind [^{3}H]epibatidine and a number of nicotinic receptor agonists with high affinity (nM) and nicotinic receptor antagonists with a lower affinity (μM). The greatest pharmacological difference between the two populations is the affinity for the α-conotoxin MII, which inhibits binding to α6-containing receptors and not that to β3-containing receptors. We also searched for the presence of the β3 subunit associated with the α-bungarotoxin binding subunits α7 and/or α8 in retina and chick brain. Immunoprecipitation studies using anti-β3 antibodies did not detect any specific α-bungarotoxin labeled receptors, thus, indicating that the β3 subunit is not present in the α-bungarotoxin receptors of these areas.

Keywords: Nicotinic acetylcholine receptor subtype; Epibatidine; α-Bungarotoxin

1. Introduction

Neuronal nicotinic acetylcholine receptors are widely expressed in the vertebrate nervous system, where they function as postsynaptic receptors to excite neurons or presynaptic receptors to modulate neurotransmitter release (Role and Berg, 1996; Wonnacott, 1997). These receptors are a family of acetylcholine-gated cation channels consisting of different subtypes, each of which has a specific anatomical distribution in the central (CNS) and peripheral nervous system (Sargent, 1993; Gotti et al., 1997a; Lindstrom, 2000).

Eight vertebrate α-subunits (α2–α9) and three β subunits (β2–β4) have been cloned. When individually expressed in heterologous systems, the α7, α8, and α9 subunits form functional homomeric channels that are blocked by the snake toxin α-bungarotoxin, whereas the α2, α3, α4 and α6 subunits form channels only when coexpressed in combination with the β2 or β4 subunits (heteromeric channels), and these channels are not blocked by α-bungarotoxin (McGehee and Role, 1995; Role and Berg, 1996; Lindstrom, 2000).

Neither the α5 nor the β3 subunits can form functional channels when coexpressed heterologously together with another α or β subunit, which is why they were referred to as orphan subunits for a long time (Sargent, 1993; Role and Berg, 1996). The participation of the α5 subunit in the formation of acetylcholine-activated channels has recently been demonstrated. It forms functional channels in oocytes only when coexpressed with the α4 and β2 (Ramirez-

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Latorre et al., 1996; Fucile et al., 1997), or with the α3 and β2 or α3 and β4 subunits, but not when expressed alone (Wang et al., 1996).

β3 subunit mRNA is expressed in several areas of the mammalian CNS, where it extensively colocalizes with the α6 subunit (Le Novère et al., 1996). It has recently been reported that a mutated form of the human β3 subunit ($\beta3^{V273T}$) can be coexpressed in oocytes with the human α3 and β4 subunits to form functional channels whose pharmacological and biophysical properties are different from those of the α3β4 combination (Groot-Kormelink et al., 1998). It has also been reported that the β3 subunit can co-assemble in oocytes with a mutated form of the α7 subunit ($^{L247T}\alpha7$), an assembly that changes the functional and pharmacological profile of $^{L247T}\alpha7$ receptors (Palma et al., 1999).

Identifying the subunit composition of native neuronal nicotinic acetylcholine receptor subtypes is a difficult but worthwhile task, since recent results have shown that the functional and pharmacological properties of heterologous subtypes may be influenced by the types of cells in which they are expressed (Lewis et al., 1997). This means that it is difficult to deduce the pharmacological properties of native receptors from those obtained in heterologous systems. The only biochemical data on the presence of the β3 subunit in neurons have been obtained for the rat cerebellum, in which it has been found that the β3 subunit is present in oligomeric receptors together with the α4, β2, and β4 subunits (Forsayeth and Kobrin, 1997).

In order to investigate the role of the β3 subunit in native chick receptors, we immunopurified β3-containing receptors using subunit-specific antibodies. We decided to use chick retina as a source of β3 subunits because previous work by Hernandez et al. (1995) has shown that β3 mRNAs are undetectable in most chick brain compartments but relatively abundant in the developing retina and trigeminal ganglia.

2. Materials and methods

2.1. Subunit-specific antibodies

Polyclonal antibodies against the α2, α3, α4, α5, α6, α7, α8, β2, β3, β4 peptides were raised as previously described (Vailati et al., 1999). Two different peptides were chosen for all of the subunits: one located in the cytoplasmic loop between M3 and M4 (CYT), and the other located at the COOH terminal (COOH). The antibodies raised against the peptides were purified on an affinity column made by coupling the corresponding peptide to CNBr-activated Sepharose 4B (Pharmacia) according to the manufacturer's instructions.

The anti-peptide serum titers were evaluated by means of enzyme-linked immuno sorbant assay (ELISA) and Western blots of the purified subtypes (Gotti et al., 1998). The serum antibodies were specific only for their respective immunizing peptide in the ELISA, and immunoprecipitation and immunolabeling were specifically inhibited only by the peptide used for immunization.

2.2. Expression of the chicken β3-specific intracellular protein

The M15 *Escherichia coli* strain containing a rep4 plasmid overproducing the lac repressor was transformed with a plasmid bearing a DNA fragment encoding part of the cytoplasmic loop of the chick β3 subunit (Met^{308}–Ala^{372}) (a generous gift of Dr. Marc Ballivet, Geneva). In order to express the recombinant protein, liquid bacterial cultures were induced by the addition of 1 mM isopropyl-β-D-thiogalactoside for 3 h. The bacteria were harvested by means of centrifugation at 3000 rpm for 20 min at 4°C, and the pellets were then frozen in liquid N_2 and analyzed in Western blots with anti-β3 antibodies.

2.3. Subtype purification

Retinas and brain regions from 1-day old chicks were dissected, immediately frozen in liquid nitrogen and then stored at −80°C. The 2% Triton X-100 retina extract was prepared as previously described (Gotti et al., 1994e,1997b). In order to bind the α6-containing receptors, the retina extract was incubated three times with 5 ml of Sepharose-4B bound to anti-α6 antibodies. The bound receptors were eluted with 100 μM of the peptide used for anti-α6 antibody production, as previously described (Gotti et al., 1994e,1997b).

The material that passed through the anti-α6 antibody column was then incubated with anti-β3 antibodies to remove the residual β3-containing receptor. The bound receptors were eluted with 100 μM of the β3 peptide used for anti-β3 antibody production (β3-containing receptors).

Recovery was determined by means of [^{3}H]epibatidine binding and quantitative immunoprecipitation of the receptors present in the solution before and after each immunopurification step, as previously described (Gotti et al., 1994a,1997b).

The brain tissue extracts were prepared as previously described (Gotti et al., 1994b).

2.4. Binding assay and pharmacological experiments on immunoimmobilized α6 and β3-containing receptors

The affinity-purified anti-α6 and anti-β3 antibodies were bound to microwells (Maxi-Sorp, Nunc) by means of overnight incubation at a concentration of 10 μg/ml in 50 mM phosphate buffer pH 7.5 at 4°C. On the following day, the wells were washed in order to remove excess unbound antibodies, and then incubated overnight at 4°C with

200 μl of 2% Triton X-100 retina membrane extract containing 100–200 fmol of [^{3}H]epibatidine binding sites: the total extract was added to the wells plated with anti-α6 antibodies, whereas the extract added to the wells coated with anti-β3 antibodies was depleted of the α6-containing receptors. The immunodepletion of the extract was performed as described above. After overnight incubation with the extract, the wells were washed and the presence of immobilized receptors was revealed by means of [^{3}H]epibatidine binding.

2.5. Immunoprecipitation of [^{3}H]epibatidine-labeled purified subtypes by anti-subunit specific antibodies

The purified α6 and β3-containing subtypes were eluted from the immunoaffinity columns by means of competition with the peptides used for the production of the antibodies. After extensive dialysis, the receptors were labeled with 2 nM [^{3}H]epibatidine and incubated overnight with a saturating concentration of affinity-purified IgG (20–30 μg). Enough goat anti-rabbit IgG were added in order to precipitate all of the immunoglobulins present in the samples, which were left for 2 h at room temperature and then centrifuged for 15 min in a microcentrifuge (10 000 g). The pellets were washed twice, using wash buffer plus 0.1% Triton X-100, and then counted by means of a β counter. The level of antibody immunoprecipitation was expressed as the percentage of immunoprecipitated [^{3}H]epibatidine-labeled receptors, taking the amount of receptors present in the solution before immunoprecipitation as 100%.

2.6. Binding assay and pharmacological experiments

(±)[^{3}H]epibatidine with a specific activity of 54.6 C/mmol was from Amersham and non-radioactive epibatidine was from RBI. The non-radioactive α-bungarotoxin and all of the cholinergic ligands are from Sigma.

The binding and pharmacological experiments were performed as previously described (Vailati et al., 1999).

3. Results

3.1. Characterization of the anti-β3 antibodies

The antibodies raised against peptides of the β3 subunit were tested on a fusion protein of the chick β3 subunit expressed in bacteria containing the sequence 308–372 of the intracellular loop of the subunit itself.

Fig. 1 shows the Western blot analysis of the bacteria homogenate obtained before (lanes 1 and 3) and after (lanes 2 and 4) induction with isopropyl-β-D-thiogalactoside. Only the antibodies directed against the intracellular peptide (KGHVDRYSFSDTEEKETTLKSKLPG) were able to recognize a single peptide of the expected molecular weight in the induced bacteria.

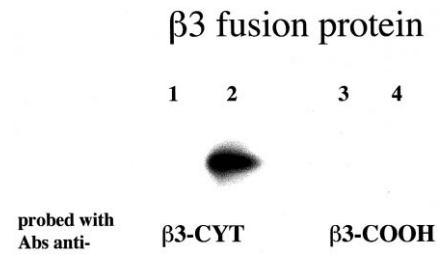

Fig. 1. Western blot analysis of the β3 fusion protein (Met^{308}–Ala^{372}) using anti-β3 antibodies. The total bacteria homogenate overexpressing the β3 subunits was separated on 15% acrylamide SDS gels, electrotransferred to nitrocellulose, and then probed with 5–10 μg/ml of anti-β3 CYT (lanes 1 and 2) and anti-β3 COOH (lanes 3 and 4). Lanes 2 and 4 contain homogenates of isopropyl-β-D-thiogalactoside-induced bacteria and lanes 1 and 3 of uninduced bacteria. The bound antibodies were revealed by means of [^{125}I]Protein A.

We then tested the anti-β3 antibodies for their ability to immunoprecipitate the receptors present in the chick retina (which is known to have the highest level of the β3 transcript) and three different areas of the brain (optic lobe, forebrain, and cerebellum) which have no detectable signal for the β3 transcript. We also tested these tissues for the presence of α6-containing receptors, because the α6 subunit colocalizes with the β3 subunit in mammalian brain, and β2 and β4-containing receptors, because we have previously shown that their content is different in chick retina and brain (Vailati et al., 1999).

The immunoprecipitation studies were performed on 2% Triton X-100 extract obtained from the corresponding tissues as described in Materials and methods. The receptors were labeled with 2 nM [^{3}H]epibatidine in the presence of 2 μM α-bungarotoxin because chick retina contains the α8 subtype, which, like other heteromeric subtypes, binds [^{3}H]epibatidine with picomolar affinity (Gerzanich et al., 1995). In order to prevent α8 binding from contributing to [^{3}H]epibatidine binding, the receptors were preincubated with α-bungarotoxin, which binds to the α8 subtype and inhibits the binding of [^{3}H]epibatidine.

Table 1 shows the results of the immunoprecipitation of anti- α6, β2, β3 and β4 in the four CNS areas. The retina was rich in α6 and β3-containing receptors, whereas the other brain regions analyzed had only a very low level of these receptors. In agreement with previously reported data (Vailati et al., 1999), we also found a high level of β4-containing receptors in the retina and a much lower level in the brain. The large majority (> 80% of the receptors) of the high-affinity [^{3}H]epibatidine receptors in the different brain areas contained the β2 subunit.

3.2. Subunit composition of the immunopurified subtypes

We used bound anti-α6 antibodies to immunopurify the α6-containing receptors. After three passages on the anti-α6 immunoaffinity column, the α6-containing receptors

Table 1
Percentage of immunoprecipitation of [^{3}H]epibatidine-labeled receptors by subunit-specific antibodies in neuronal tissues extracts
Immunoprecipitation was carried out as described in Materials and methods using saturating concentrations of anti-subunit antibodies. The results are expressed as percentages of [^{3}H]epibatidine-labeled receptors, taking the amount of receptor present in the solution before immunoprecipitation as 100%. The percentage of immunoprecipitation was subtracted from the value obtained in control samples containing an identical concentration of normal rabbit IgG. The values are the means ± SEM of three determinations.

Antibodies	Optic lobe	Forebrain	Cerebellum	Retina
Anti-α6-COOH	5 ± 1	4 ± 1	3 ± 2	34 ± 3
Anti-α6-CYT	4 ± 0.5	1 ± 0.2	1 ± 0.7	35 ± 4
Anti-β3-COOH	0.6 ± 0.2	0.2 ± 0.2	6 ± 1.5	28 ± 6
Anti-β3-CYT	5 ± 0.4	1 ± 0.3	7 ± 0.1	32 ± 2
Anti-β2-COOH	93 ± 5	77 ± 2.8	85 ± 1.2	30 ± 1
Anti-β2-CYT	89 ± 2	85 ± 2.7	80 ± 2	32 ± 1
Anti-β4-COOH	11 ± 2	16 ± 0.9	17 ± 1	76 ± 1.5
Anti-β4-CYT	13 ± 0.5	14 ± 0.8	11 ± 0.3	72 ± 2.5

decreased from 34% to less than 2% of the total number of [^{3}H]epibatidine-labeled receptors. Immunoprecipitation experiments with this immunodepleted α6 extract showed that although the number of β3-containing receptors decreased, they still represented 13 ± 1% of the [^{3}H]epibatidine-labeled receptors.

We therefore decided to use anti-β3 antibodies to immunodeplete the β3-containing receptors sequentially in order to analyze their subunit content.

The α6 and β3-containing receptors were eluted from the immunoaffinity column using the corresponding peptides and, after extensive dialysis, their subunit contents were analyzed in immunoprecipitation experiments using the antibodies directed against the different chick neuronal nicotinic acetylcholine receptor subunits.

Fig. 2 shows the results of this quantitative immunoprecipitation. As can be seen in the upper part of the figure, almost all of the α6-containing receptors contained the β4 subunit, 40% the α3 subunit, and more than 50% the β3 subunit. No α2, α4, α5, α7 or α8 subunits were found and only 7.5% of the receptors contained the β2 subunit.

The subsequently purified β3-containing receptors were very heterogeneous in their subunit content for both the α and β subunits. As shown in the lower part of Fig. 2, the anti- α2, α3, α4, α5, α6, α7, α8, β2, β3 and β4 antibodies immunoprecipitated 26 ± 1, 35 ± 3, 33 ± 4, 1.5 ± 0.9, 1.5 ± 0.5, 1.5 ± 1, 1.5 ± 0.5, 45.2 ± 4, 20.3 ± 0.8 and 41.7 ± 2% of the [^{3}H]epibatidine-labeled receptors, respectively.

The partial immunoprecipitation obtained with the anti-α2, α3, α4, β2 and β4 antibodies was due to the heterogeneous subunit composition of these receptors, and not to an inability to immunoprecipitate the receptors, because the same antibodies could immunoprecipitate more than 70% of the corresponding subunit-containing receptors purified from other nervous system regions (data not shown). In the case of the anti-β3 antibodies, the partial immunoprecipitation was probably due to the fact that antibodies directed against the same epitope (β3-CYT) were used for both immunopurification and immunoprecipitation, and it is thus possible that incomplete dialysis of the peptide used for the elution may have led to underestimation of the extent of immunoprecipitation.

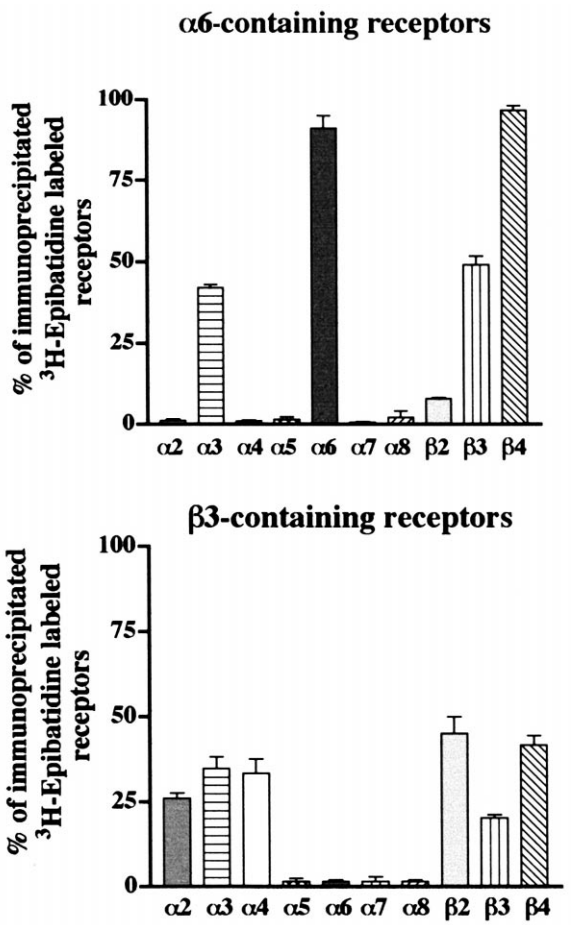

Fig. 2. Immunoprecipitation of sequentially immunopurified α6 and β3-containing receptors from chick retina. The α6-containing receptors were immunopurified by passing three times, a 2% retina Triton X-100 extract through an immunoaffinity column with bound anti-α6 antibodies (α6-containing receptors). The material that passed through this column was devoid of α6-containing receptors and was then passed through a β3 affinity column (β3-containing receptors). Both bound receptors were eluted from the affinity column using 100 μM of the peptide used for antibody production. After extensive dialysis to remove the peptides, the α6 and β3-containing receptors were labeled with 2 nM [^{3}H]epibatidine and immunoprecipitation was carried out as described in Materials and methods using saturating concentrations of anti-subunit antibodies. The results are expressed as percentages of [^{3}H]epibatidine-labeled receptors, taking the amount of receptor present in the solution before immunoprecipitation as 100%. The percentage of immunoprecipitation was subtracted from the value obtained in control samples containing an identical concentration of normal rabbit IgG. The values are the means ± SEM of three determinations.

These immunoprecipitation studies indicate that there are at least two populations of β3-containing receptors in chick retina present in a ratio of 3:1. In the first population, the β3 subunit is associated with the α6 and β4 subunits; in the second, it is associated with the α2, α3, α4, β2 and β4 subunits and not with the α6 subunit. We did not investigate all of the possible subunit combinations of this second population because of the low recovery of β3-containing receptors.

3.3. Pharmacological characterization of the α6 and β3-containing receptors

[^{3}H]epibatidine binds to α6 receptors at a single high-affinity site with a K_d of 35 pM (Vailati et al., 1999). The pharmacological profile of the α6 receptors was characterized by testing the relative efficacy by which various cholinergic agonists and antagonists inhibited the binding of 0.1 nM [^{3}H]epibatidine at equilibrium. It was found that the relative efficacy of the agonists in the competition experiments was epibatidine ≫ cytisine > nicotine > 1,1-dimethyl - 4 - phenylpiperazinium > acetylcholine > carbamylcholine and except for carbamylcholine, all of them had relatively low K_i values (in the low nanomolar range). The rank order of antagonist potency was α-conotoxin MII > methyllycaconitine > dihydro-β-erythroydine > MG624 > D-tubocurarine > decamethonium > hexamethonium. We found that the α-conotoxin MII, a compound described as an antagonist of the rat α3β2 subtype, was the most potent drug (K_i = 66 nM) in competing for α6 receptors (Table 2).

β3-containing receptors also bound [^{3}H]epibatidine with high affinity; the K_d of the binding calculated from six separate experiments was 9 pM with a coefficient of variation (CV) of 16%. Fig. 3 shows a typical saturation curve of the binding of [^{3}H]epibatidine to β3-containing receptors, which is consistent with the presence of a single class of high-affinity binding sites, as is also suggested by the linearity of the Scatchard analysis.

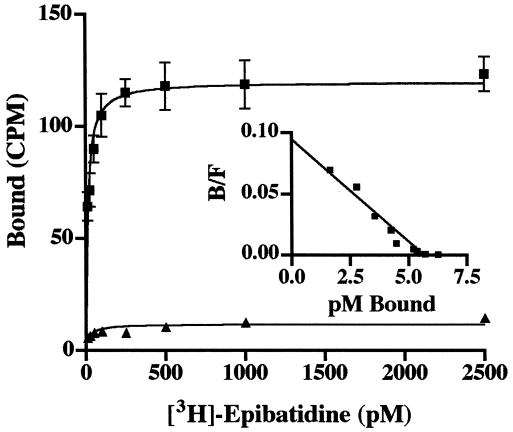

Fig. 3. Saturation curve of [^{3}H]epibatidine binding to immunoimmobilized β3 receptors, and its Scatchard analysis (insert). To measure total binding, the immunoimmobilized receptors were incubated overnight at 4°C with the indicated concentrations of [^{3}H]epibatidine; a specific binding was measured also in the presence of 100 nM epibatidine. The total (■) and a specific binding (▲) shown is that obtained from a representative experiment; the K_d value of 9 pM [coefficient of variation (CV) = 16%] was calculated by simultaneously fitting data from six separate experiments. The Scatchard plot of the saturation curve shows the presence of a single class of high-affinity sites.

We also analyzed the pharmacological profile of the β3-containing receptors by performing competition binding experiments at equilibrium with several nicotinic receptor agonists and antagonists. Fig. 4 shows the inhibition curves of the acetylcholine receptor agonists (part A) and antagonists (part B). The K_i values of the inhibition curves obtained by simultaneously fitting the data of three–four different experiments are shown in Table 2, together with the K_i values of the same drugs for the α6-containing receptors.

Apart from the agonist carbamylcholine, the β3-containing receptors bound a number of nicotinic agonists with nanomolar affinity, the order of potency being epibatidine > cytisine > acetylcholine > 1,1-dimethyl-4-phenylpiperazinium > nicotine > carbamylcholine. They had a much lower affinity for the nicotinic antagonists (K_i values in the micromolar range), with an order of potency of F3 > dihydro-β-erythroydine > methyllycaconitine > D-tubocurarine > MG624 > α-conotoxin MII > decamethonium. The major pharmacological difference between the α6 and β3-containing receptors was the K_i value for the α-conotoxin MII, which did not inhibit [^{3}H]epibatidine binding to the β3-containing receptors at concentrations up to 5 μM. The toxin methyllycaconitine also inhibited binding to the β3-containing receptors but at a higher concentration (K_i of 5.3 μM) than it inhibited binding to the α6-containing receptors.

Table 2
Pharmacological characterization of immunoimmobilized subtypes
The K_d and K_i values were derived from [^{3}H]epibatidine saturation and competition binding curves for the α6- and β3-containing subtypes. The curves obtained from three separate experiments were fitted using a non-linear least-squares analysis program and the *F*-test (Gotti et al., 1998). The numbers in brackets represent the percentage of CV.

Ligand	α6 (K_i nM)*	β3 (K_i nM)
Cytisine	11 (36)	9 (28)
Nicotine	20 (31)	86 (37)
Dimethyl-4-phenylpiperazinium	31 (37)	73 (27)
Acetylcholine	76 (26)	48 (26)
Carbamilcholine	975 (29)	3600 (35)
α-Conotoxin MII	66 (24)	> 5000
Methyllycaconitine	1350 (25)	5300 (17)
Dihydro-β-erythroydine	2800 (13)	3600 (23)
MG624	4520 (26)	7900 (27)
D-tubocurarine	7700 (18)	6600 (22)
Decamethonium	35900 (16)	16000 (41)
F3	1600 (26)	3000 (27)
[^{3}H]epibatidine (K_d pM)	35 (18)	9 (16)

* Taken from Vailati et al., 1999.

3.4. β3 subunit and α-bungarotoxin receptors

Recent electrophysiological data have shown that the β3 subunit is able to associate with a mutated form of the α7 subunit (^{L247T}α7) to form channels whose electrophysiological and pharmacological properties are different from those of the homomeric ^{L247T}α7 receptors (Palma et al., 1999). In order to test whether the β3 subunit is associated with the α-bungarotoxin binding receptors in the chick CNS, we performed immunoprecipitation experiments with chick retina, optic lobe, cerebellum and forebrain using the anti-α7, anti-α8 and anti-β3 antibodies. The results are shown in Table 3.

The anti-α7 and anti-α8 antibodies immunoprecipitated most of the α-bungarotoxin receptors with immunoprecipitation values very similar to those previously reported by Keyser et al. (1993) and our group (Gotti et al., 1994b), whereas the anti-β3 antibodies only immunoprecipitated 1–2% of the receptors, a value that indicates a lack of co-assembly of β3 subunit with the α7 or α8 subunits in the α-bungarotoxin receptors.

Table 3
Percentage of [^{125}I]α-bungarotoxin-labeled receptors immunoprecipitated by subunit-specific Abs in neuronal tissue extracts
Immunoprecipitation was carried out as described in Table 1 using extracts labeled with 10 nM [^{125}I]α-bungarotoxin and saturating concentrations of anti-subunit antibodies. After overnight incubation, the complexes were immunoprecipitated by means of goat anti-rabbit IgG.

Antibodies	Optic lobe	Forebrain	Cerebellum	Retina
Anti-α7	96±8	84±5	80±2	35±5
Anti-α8	26±3.4	15±1	13±0.6	76±7
Anti-β3	1±0.9	0.5±0.5	1±0.3	0.9±0.8

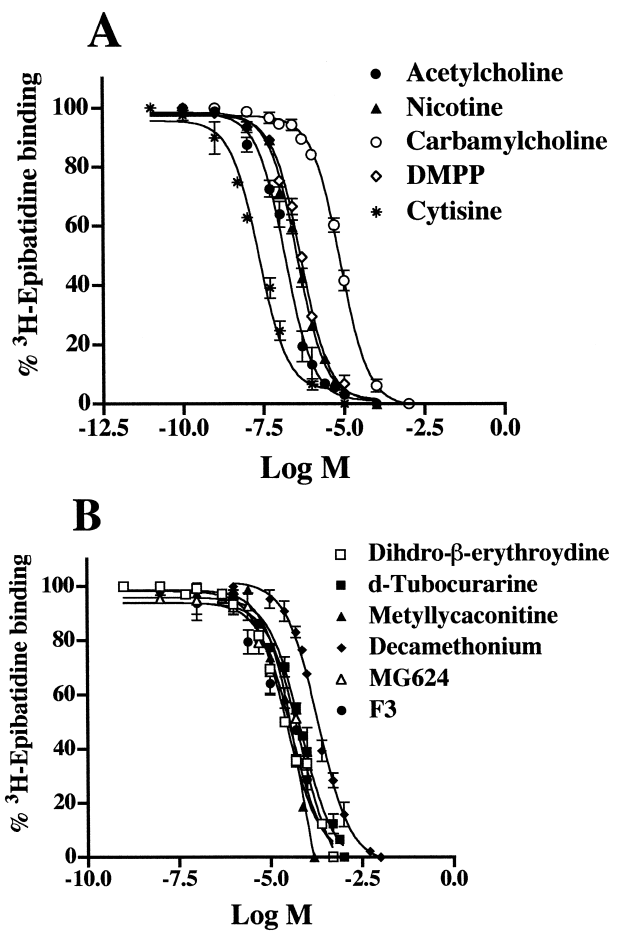

Fig. 4. Inhibition of [^{3}H]epibatidine binding to immunoimmobilized β3 receptors by nicotinic receptor agonists (A) and antagonists (B). The β3-containing receptors immunoimmobilized on the anti-β3 CYT antibodies (as described in Materials and methods) were preincubated for 30 min at 20°C with the indicated concentrations of nicotinic ligands; [^{3}H]epibatidine was then added at a final concentration of 100 pM, and the mixture was left overnight at 4°C. The curves were obtained by fitting data from three separate experiments using the LIGAND program. In each experiment, each dilution of the drug was tested in triplicate. All of the values are expressed in relation to [^{3}H]epibatidine specific binding to the receptors (considered as 100%).

Affinity chromatography of the α-bungarotoxin bound to Sepharose resin was used to purify the α-bungarotoxin receptors present in chick retina and optic lobe, which were then subfractionated using the subunit-specific anti-α7 and anti-α8 antibodies. After immunopurifying, the α7 subtype from the optic lobe and the α8 subtype from the retina as previously described (Gotti 1994a,1997b), we probed them for the presence of the β3 subunit, but could not determine any specific labeling (data not shown).

4. Discussion

There is little information concerning the distribution, role, and function of the β3-containing nicotinic receptors. It has only recently been shown that a mutated form of the β3 subunit is able to form functional channels. The only previously available data concerned its cloning (Deneris et al., 1989; Hernandez et al., 1995), its mRNA distribution in chick and rat brain (Hernandez et al., 1995; Le Novère et al., 1996) and its presence with the α4, β2, and β4 subunits in oligomeric receptors expressed in rat cerebellum (Forsayeth and Kobrin, 1997).

We have devised an alternative approach for studying the subunit composition of chick native neuronal receptors and raised a series of antibodies that specifically recognize all of the known subunits (at least two antibodies recognizing different parts of each subunit). These antibodies can be used to immunopurify and/or immunoimmobilize the subtypes containing the desired subunit, and thus, to investigate the pharmacological and functional properties of the subtypes by means of binding studies or after reconstitution in lipid bilayers. This last technique allows the purification of very clean receptor subtypes, and has the advantage of making it possible to study the properties of native wild-type neuronal receptors.

Using these subunit-specific antibodies, we found that chick retina contained high levels of the β3 subunit in at least two receptor populations: one containing the α6 and β4 subunits (and possibly also the α3 subunit) and the other containing the α2, α3, α4, β2 and β4 subunits and not the α6 subunit. Both populations of β3-containing receptors bound nicotinic receptor agonists with high affinity and, although the order of potency of the agonists in the two subtypes was different (epibatidine ≫ cytisine > nicotine > 1,1-dimethyl-4-phenylpiperazinium > acetylcholine > carbamylcholine in the α6-containing receptors and epibatidine > cytisine > acetylcholine > 1,1- dimethyl -4-phenylpiperazinium > nicotine > carbamylcholine for the non-α6-containing receptors, the absolute K_i values were not statistically different.

The K_i values determined for a number of old (D-tubocurarine, dihydro-β-erythroidine) and new (MG624 and F3) nicotinic antagonists (see Gotti et al., 1998) were also very similar, the only major pharmacological difference being the different affinity of the α-conotoxin MII and the toxin methyllycaconitine for the two populations. Both α-conotoxin MII and methyllycaconitine bound with high affinity to the α6-containing receptors but α-conotoxin MII did not bind to the β3-containing receptors and the affinity of methyllycaconitine was less than that for the α6-containing receptors. α-Conotoxin MII is a toxin purified from the snail *Conus* and selectively blocks the oocyte-expressed rat α3β2 subtype at nanomolar concentrations but it is ineffective in other rat subtypes (Cartier et al., 1996). Our results suggest that α-conotoxin MII can interact differently with chick receptor subtypes and suggest that it is not the presence of the β3 subunit that determines the high affinity for α-conotoxin MII but rather the presence of the α6 and/or β4 subunits. However, binding studies of transfected cell lines expressing the chick receptor subtypes are needed in order to clarify this point.

It has been recently reported that the β3 subunit can form a heteromeric channel with a mutated form of the α7 subunit that binds α-bungarotoxin (Palma et al., 1999). We therefore tested whether such an association is present in vivo by performing immunoprecipitation experiments on the α-bungarotoxin receptors in the retina and three different brain areas using anti-β3 antibodies. We did not find any specific immunoprecipitation of α-bungarotoxin receptors in any tissue at any developmental time other than that obtained using anti-α7 and/or anti-α8 antibodies. These results indicate that there is no β3 subunit in the α-bungarotoxin receptors in these areas.

Since our assay is based on α-bungarotoxin binding to receptors, our conclusions are not valid if the α7 and α8 subunits assemble with other subunits and change their affinity for α-bungarotoxin in such a way that they are no longer able to bind α-bungarotoxin with high affinity. However, although this possibility may be valid for the association of α7 and α8 subunits with other subunits, it does not seem to apply in the case of the association of the β3 subunit with the α7 subunit because α-bungarotoxin is still able to bind and block heteromeric ^{L247T}α7β3 receptors (Palma et al., 1999).

It is very difficult to study neuronal nicotinic acetylcholine receptors not only because of their in situ heterogeneity and complexity, but also because of their many and complex functional roles. Our immunoprecipitation studies have revealed that the β3 subunit is present in at least two distinct receptor populations in the retina, but this is only the first step towards determining their precise cellular distribution and understanding their physiological role.

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