0026-895X/99/010011-09$3.00/0

MOLECULAR PHARMACOLOGY, **56:**11–19 (1999).

Functional α6-Containing Nicotinic Receptors Are Present in Chick Retina

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Received January 11, 1999; accepted April 15, 1999

This paper is available online at http://www.molpharm.org

ABSTRACT

Despite the fact that the neuronal chick α6 subunit was first cloned several years ago and recently has been shown to form acetylcholine (ACh)-activated channels in heterologous systems, no information is yet available concerning the structure and function of the α6-containing nicotinic receptors in neuronal tissues. Using subunit-specific antibodies directed against two different epitopes of the chick α6 subunit, we performed immunoprecipitation experiments on immunopurified α6-containing receptors radiolabeled with the nicotinic agonist [^{3}H]epibatidine (Epi): almost all of the α6 receptors contained the β4 subunit, 51% the β3 subunit, 42% the α3 subunit, and 7.5% the β2 subunit. Western blot analyses of the purified receptors confirmed the presence of the α3, β3, β2, and β4 subunits, and the absence of the α4, α5, and α7 subunits. The α6-containing receptors bind [^{3}H]Epi (K_d = 35 pM) and a number of other nicotinic agonists with very high affinity, the rank order being Epi ≫ cytisine > nicotine > 1,1-dimethyl-4-phenylpiperazinium > acetylcholine > carbamylcholine. The α6 receptors also have a distinct antagonist pharmacological profile with a rank order of potency of α-conotoxin MII > methyllycaconitine > dihydro-β-erythroydine > MG624 > *d*-tubocurarine > decamethonium > hexamethonium. When reconstituted in lipid bilayers, the α6-containing receptors form functional cationic channels with a main conductance state of 48 pS. These channels are activated by nicotinic agonists in a dose-dependent manner, and blocked by the nicotinic antagonist *d*-tubocurarine.

Neuronal nicotinic acetylcholine receptors (nAChRs) are a family of ligand-gated ion channels that play a role in central and peripheral nervous systems under both normal and pathological conditions (Dani and Heinemann, 1996; Gotti et al., 1997a; Léna and Changeux, 1997; Wonnacott, 1997). Eleven genes coding for the nAChR subunits α2 to α9 and β2 to β4 have so far been identified. Although there are many subtypes consisting of different subunit combinations, two main classes of nicotinic receptors can be identified on the basis of their function and pharmacology: homomeric channels, that are all blocked by the competitive antagonist α-bungarotoxin (αBgtx), and heteromeric channels that are completely insensitive to it (reviewed in Sargent, 1993; Role and Berg, 1996). Heterologous expression studies have demonstrated that homomeric channels can only be formed by the α7, α8, and α9 subunits, whereas the heteromeric channels can be formed by the α2, α3, or α4 subunits combined with the β2 or β4 subunits, as well by the coexpression of α5 or β3 with another β and α subunit (other than the α subunits forming homomeric channels) (McGehee and Role, 1995; Ramirez-Latorre et al., 1996; Groot-Kormelink et al., 1998).

Although heterologously expressed nAChR subtypes have provided a lot of information concerning the functional diversity of nAChR subtypes, recent data have clearly shown that channels with different pharmacological and biophysical properties can be obtained depending on the expression system used (Fucile et al., 1997; Lewis et al., 1997), and biochemical and immunological experiments in vertebrate brain and ganglia have suggested that native nAChRs may be more complex than previously thought (Conroy and Berg, 1995; Forsayeth and Kobrin, 1997).

This work was supported in part by grants from Fabriques de Tabac Réunies, Neuchâtel, Switzerland, the Italian Ministry of University and Scientific and Technological Research, the European Program "Training and Mobility of Researchers," Contract ERB4061PL97–0790 and the Telethon Grant 1047 to C.G.

ABBREVIATIONS: nAChR, neuronal nicotinic acetylcholine receptor; αBgtx, α-bungarotoxin; ACh, acetylcholine, Carb, carbamylcholine; COOH, subunit COOH peptide; Cyt, cytisine; CYT, subunit cytoplasmic peptide; Epi, epibatidine; DMPP, 1,1-dimethyl-4-phenylpiperazinium; Nic, nicotine; MLA, methyllycaconitine; DHβE, dihydro-β-erythroidine; MII, α-conotoxin MII; *d*-TC, *d*-tubocurarine; COL, chick optic lobe; Abs, polyclonal antibodies; mAbs, monoclonal antibodies.

Among the nAChR subunits cloned so far, α6 (Lamar et al., 1990) has long been considered an "orphan" subunit, because it was impossible to obtain any α6-containing functional channels. The amino acid sequence of the chick α6 subunit is most closely related to that of α3 subunit (66.5% identity). In rat brain, α6 mRNA is selectively concentrated in catecholaminergic nuclei where it is colocalized with β3 mRNA (Le Novère et al., 1996); in the chick nervous system, α6 mRNA is abundantly expressed in neuroretina (where it coexists with α3, β2, and β4 mRNAs) but not in the optic tectum or peripheral ganglia (Fucile et al., 1998).

It has only very recently been demonstrated that the chick α6 subunit forms a functional channel when expressed with the human β4 subunit in oocytes (Gerzanich et al., 1997) and can form functional receptors in human BOSC 23 cells when it is coexpressed with either the chick β2 or β4 subunits or with both the β4 and α3 subunits together (Fucile et al., 1998).

Because no information is available concerning the structure and function of the α6-containing channels present in neuronal tissues, we decided to immunopurify this receptor subtype from chick retina. This article reports the purification and characterization of this receptor, its subunit composition and pharmacology, and its functional behavior after reconstitution in planar lipid bilayers.

Materials and Methods

Antibody Production and Characterization

The monoclonal antibody (mAb) 270 raised against chicken brain nAChR and directed against the β2 subunit (Whiting et al., 1987) was a generous gift of Dr. J. Lindstrom, University of Pennsylvania, Philadelphia, PA. The mAb 35 raised against the muscle-type AChR recognizes the α1 subunit and cross-reacts with the α5 and α3 subunits (Conroy et al., 1992, 1998), was purified from hybridoma cell line obtained from the American Type Culture Collection (Rockville, MD). The mAb 299, raised against rat brain nAChR and directed against the α4 subunit (Whiting and Lindstrom, 1988), as well as the mAb 313 raised against the fusion protein containing the putative cytoplasmic of the α3 subunit (Whiting et al., 1991), were both purchased from Research Biochemicals Inc. (Natick, MA).

The polyclonal Abs (Abs) against the α3, α4, α5, α6, α7, α8, β2, and β4 peptides were raised as previously described (Gotti et al., 1994); their peptide sequences are shown in Table 1. 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 cyanogen bromide-activated Sepharose 4B (Pharmacia Inc., Uppsala, Sweden) according to the manufacturer's instructions.

For the β3 protein, a polyclonal antiserum was raised in rabbit using a fusion protein (the generous gift of Dr. Marc Ballivet, University of Geneva, Geneva, Switzerland) located in the extracellular part of the β3 subunit between S105 and I180.

The antipeptide serum titers were evaluated by means of enzyme-linked immunoassay and Western blots of the purified subtypes (Gotti et al., 1998; Maggi et al., 1999). The serum antibodies were specific only for their respective immunizing peptide in enzyme-linked immunoassay, and each immunoprecipitation and immunolabeling was specifically inhibited only by the peptide used for the immunization.

In addition, to exclude cross-reactivity between the anti-α3 and anti-α6 Abs, the anti-α3 COOH, and anti-α3 CYT polyclonal antibodies were also respectively passed through columns with bound α6 COOH or α6 CYT peptides, and the anti-α6 COOH and anti-α6 CYT Abs through columns with bound α3 COOH or α3 CYT peptides.

α6 Subtype Immunopurification

The chick optic lobe (COL) and retina were dissected from 1-day-old chicks, immediately frozen in liquid nitrogen, and then stored at −80°C. The retina and COL extracts were prepared as previously described (Gotti et al., 1991, 1997b). To bind the α6-containing receptors, the retina extract was incubated three to four times with 5 ml of Sepharose-4B bound to anti-α6 Abs. The bound receptors were eluted with 0.2 M glycine (pH 2.2) or 100 μM of the corresponding peptide used for Ab production as previously described (Gotti et al., 1991, 1997b).

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

Binding Assay and Pharmacological Experiments on Immunoimmobilized α6 Receptors

The affinity-purified anti-α6 Abs were bound to microwells (MaxiSorp; Nunc, Naperville, IL) by means of overnight incubation at 4°C at a concentration of 10 μg/ml in 50 mM phosphate buffer, pH 7.5. On the following day, the wells were washed to remove the excess of unbound Abs, and then incubated overnight at 4°C with 200 μl of 2% Triton X-100 retina membrane extract containing 100 to 200 fmol of [^{3}H]Epi binding sites.

[^{3}H]Epi Binding to Solubilized Receptor. Like Gerzanich et al. (1995), we found that [^{3}H]Epi binds with high affinity to the β2- and β4-containing subtypes (picomolar affinity), but it also binds to the α7 subtypes with a low nanomolar affinity and to the α8-containing receptor with picomolar affinity. To ensure that the α7 and α8 subtypes did not contribute to [^{3}H]Epi binding during the α6 receptors purification, all the binding and immunoprecipitation experiments were performed in the presence of 2 μM αBgtx, which specifically binds to the α7 and α8 subtypes and blocks [^{3}H]Epi binding.

The Triton X-100 extract of retina and COL membranes was assayed using DE52 ion-exchange resin (Whatman). Aliquots of the extract (50–100 μl) or purified receptors eluted with the peptides (dialyzed against 10 mM Na Phosphate 10 mM pH 7.4, 50 mM NaCl, and 0.1% Triton X-100, wash buffer) were incubated overnight at 4°C with 2 nM [^{3}H]Epi. The incubation mixture was diluted to 200 μl with H_2O and applied to a 500-μl DE52 column. After being washed with 10 ml of wash buffer to remove the unbound [^{3}H]Epi, the bound receptor was eluted with 2 N NaOH and counted. The nonspecific

TABLE 1
Amino acid sequences of peptides used to produce subunit-specific polyclonal Abs

α3	cQPLMTGDDM (COOH terminal)[a]
α3	TSDEENNQKPKPFYTSEFSNLNC (CYT loop)
α4	cgPPWLAGMI (COOH terminal)
α4	PNFTTSSSPSPQSNEPSPTSSFC (CYT loop)
α5	cPVHIGSTNT (COOH terminal)
α5	cVDRYFAQKEEKGNMSGSESSRNTLEA (CYT loop)
α6	cgIQPLIADT (COOH terminal)
α6	ISKKTKKGSAKTSGKSKHSKHKDNKLHKEQRC (CYT loop)
α7	yCSPTEEENLLHSGHPSEGDPDL (CYT loop)
β2	cQLGQGTPTSK (COOH terminal)
β2	AKAEGLNGYRERQGQGPDPPAPCG (CYT loop)
β3	cgQMWLNSTL (COOH terminal)
β3	KGHVDRYSFSDTEEKETTLKSKLPGc (CYT loop)
β4	cQNHIAATNP (COOH terminal)
β4	PENNSPRQKPANCKKTRAENLC (CYT loop)

[a] Amino acid sequences of peptides used to produce subunit-specific polyclonal Abs. Capital letters represent amino acids present in subunit sequence, whereas lowercase letters indicate extra-sequence amino acid introduced to enable specific coupling to carrier protein. Putative subunit region from which sequence was taken is indicated in parentheses.

binding of [^{3}H]Epi was determined in parallel by means of coincubation with 100 to 200 nM cold Epi.

Immunoimmobilized Subtype. The receptors immobilized by the corresponding subunit-specific Abs were incubated overnight at 20°C with 300 μl of [^{3}H]Epi at concentrations ranging from 0.005 to 5 nM. All of the incubations were performed in a buffer containing 50 mM Tris-HCl, pH 7, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, 2 mg/ml BSA, and 0.05% Tween 20. Specifically labeled ligand binding was defined as total binding minus the binding in the presence of 100 nM cold Epi. The inhibition of [^{3}H]Epi binding to the immobilized subtypes induced by the cholinergic ligands was measured by preincubating the indicated concentrations of the compounds for 30 min at room temperature, followed by overnight incubation with 0.1 nM [^{3}H]Epi.

After incubation, the wells were washed seven times with ice-cold PBS containing 0.05% Tween 20, and the bound radioactivity recovered by incubation with 200 μl of 2N NaOH for 2 h. The bound radioactivity was then determined by means of liquid scintillation counting in a beta counter for [^{3}H]Epi.

Data Analysis. The experimental data obtained from the saturation binding experiments performed in membrane or solubilized receptors were analyzed by means of a nonlinear least square procedure using the LIGAND program as described by Munson and Rodbard (1980). The calculated binding parameters were obtained by simultaneously fitting 10 independent experiments.

The selection of the best fitting (i.e., one-site versus two-site model) and the evaluation of the statistical significance of the parameters (i.e., comparison of the binding parameters of the two groups), were based on the F test for the "extra sum of square" principle. A P value of <0.05 was considered statistically significant (Munson and Rodbard, 1980) .

The K_i values of all of the tested drugs were also determined by means of the LIGAND program using the data obtained from three independent competition experiments and compared by means of the F test as described above.

Expression of Chick α6β4 and α3β4 Subtypes in BOSC 23 Cells

Calcium phosphate-mediated transient tranfections of the α3β4 and α6β4 subtypes in the human BOSC23 cell line were carried out as previously described by Ragozzino et al. (1997) and Fucile et al. (1998).

The presence of the expressed subtypes was measured by means of [^{3}H]Epi saturation binding experiments to cell homogenates as described in Gotti et al. (1998); the K_d and B_{max} were 98 pM (CV = 14%) and 51 fmol/mg of protein for the α3β4 subtype, and 46 pM (CV = 13%) and 42 fmol/mg of protein for the α6β4 subtype.

Bilayer Formation and Subtype Insertion

The purified α6 subtype eluted from the corresponding immunoaffinity columns were dialyzed, concentrated, and stored at −20°C until used.

The purified receptors were incorporated in asolecithin liposomes (Sigma Chemical Co., St. Louis, MO) by means of dialysis, and then fused with preformed bilayers as previously described. All of the point-amplitude histograms were constructed from 5-s current fluctuation traces digitized at a sampling rate of 2000 samples/s. The current-voltage curve was constructed from all of the histograms, and the channel conductance was calculated from the linear portion of the curve. In addition, the global open-state probability (P_o) was calculated from the areas under the peaks of the histograms.

Preliminary experiments were performed by adding carbamylcholine (Carb) 1 mM to the *trans* or *cis* side of the bilayer to identify the orientation of the channels. In the reported experiments, the agonists dissolved in 150 mM NaCl and 5 mM Tris-HCl (at the concentrations given in *Results*) were applied to the side of the bilayer in which the channels have been correctly incorporated.

The 50% activation value (EC_{50}) was calculated from the plot of P_o versus [agonist] as the value of the agonist concentration necessary to obtain a level of activity midway between spontaneous activity and maximum P_o.

Further details of the experimental procedures have been previously described (Gotti et al., 1991, 1994, 1997).

Materials

Antiprotease inhibitors, asolecithin type IIS, cholinergic ligands, Triton X-100, and anti-rabbit and anti-rat antisera were purchased from Sigma; nonradioactive Epi was purchased from RBI; cyanogen bromide-activated Sepharose 4BCL was purchased from Pharmacia, Sweden; [(±)-^{3}H]Epi, ^{125}I-labeled αBgtx, ^{125}I-labeled protein A, and [^{3}H]Epi were purchased from Amersham (Buckinghamshire, UK); and the reagents for gel electrophoresis were purchased from Bio-Rad Labs (Hercules, CA).

Results

Specificity of Antisubunit Antibodies Against nAChR Subtypes Present in COL and Retina. For each subunit, we raised polyclonal antibodies against a peptide located in the cytoplasmic loop, which is the most divergent region of the otherwise homologous nAChR subunits; to have an additional control for each subunit, we also raised Abs directed against a peptide located on the COOH terminal region (see Table 1).

Given the high degree of identity between the α3 and α6 subunits (66.5%), special care was taken to detect the specificity of the anti-α3 and anti-α6 Abs. To this end, the anti-peptide Abs were tested on Western blots of human BOSC 23 cells tranfected with the α3β4 and α6β4 subtypes. Homogenates of α3β4 (Fig. 1, lane 1) and α6β4 (Fig. 1, lane 2) tranfected and/or untranfected BOSC 23 cells (Fig. 1, lane 3) were run on 7.5% SDS-polyacrylamide gel electrophoresis and then electrotransferred to nitrocellulose and tested with the anti-α3 COOH (Fig. 1A), anti-α3 CYT (Fig. 1B), anti-α6 COOH (Fig. 1C), and anti-α6 CYT (Fig. 1D). Both anti-α3 Abs only recognized the tranfected α3 and not the α6 subunits,

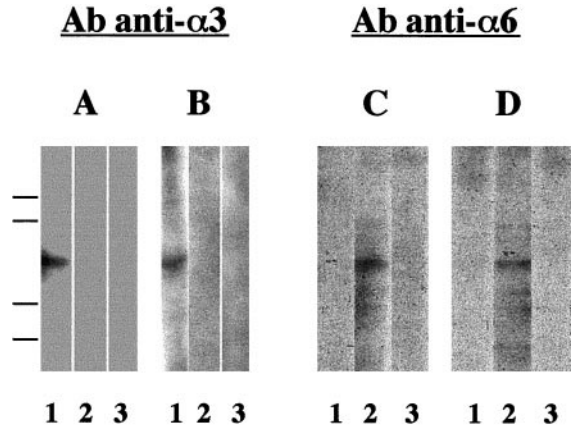

Fig. 1. Western blot analysis of human BOSC 23 cells tranfected with the α3β4 or α6β4 subtypes. Lane 1, homogenate of cells tranfected with the α3β4 subunits; lane 2, homogenate of cells tranfected with the α6β4 subunits, and lane 3, untransfected BOSC 23 cells. For each lane, 100 μg of cell homogenate was loaded and separated on 7.5% acrylamide SDS gels, electrotransferred to nitrocellulose, and then probed with 5 to 10 μg/ml of anti-α3 COOH (A), anti-α3 CYT (B), anti-α6 COOH (C), and anti-α6 CYT (D). Bound Abs were revealed by means of ^{125}I-labeled protein A. Molecular mass markers (top to bottom) are 97, 66, 45, and 31 kDa.

and the anti-α6 Abs only recognized the tranfected α6 and not the α3 subunits.

The specificity of all the Abs was tested by immunoprecipitation experiments using Triton X-100 extract obtained from COLs and retina. After solubilization, the receptors were first preincubated with 2 μM αBgtx to block the binding of [^{3}H]Epi to the α7 and α8 subtypes, and then labeled with [^{3}H]Epi and immunoprecipitated by the antibodies directed against the α3, α4, α5, α6, β2, β3, and β4 subunits. The Abs immunoprecipitated the receptors to a different extent in the two tissues, but the percentage of immunoprecipitation was very similar for the Abs directed against the same subunit and, in the case of the α3, α4, α5, and β2 subunits, also similar to that obtained using commercially available mAbs (Table 2).

From these immunoprecipitation experiments, we assumed that almost all of the heteromeric receptors in COL contain the β2 subunit, which is mainly associated with the α4 subunit but can also be found with the α5 and/or the α3 subunits. In chick retina, the majority of the [^{3}H]Epi binding receptors had the β4 subunit and a subpopulation also contains the β2 subunit, but they are very heterogeneous in terms of their α subunit expression. Both types of Abs directed against the α6 and β3 subunits recognized [^{3}H]Epi-labeled receptors in the retina to a similar and much greater extent than that determined in the chick optic tectum. This finding is in line with previously reported data that the α6 and β3 subunit mRNAs are detectable in the retina and not in the optic tectum (Hernandez et al., 1995; Fucile et al., 1998).

COL and retina also have receptors containing the α7, α8 and α7-α8 subunits that bind ^{125}I-labeled αBgtx. To have a complete profile of these Abs, we tested them for their ability to immunoprecipitate ^{125}I-labeled αBgtx receptors, but never detected any specific immunoprecipitation (data not shown).

Subunit Composition of Purified α6 Subtype. Our immunoprecipitation experiments suggested that retina would be a suitable tissue from which to purify the α6-containing receptors selectively. To this end, we thrice passed the retina extract on an affinity column with bound anti-α6 cytisine (Cyt) Abs and, after the passage, monitored depletion by means of immunoprecipitation. Selective immunodepletion of the α6-containing receptors was demonstrated by the fact that their number decreased from 34 ± 4% (retina extract) to 5 ± 3% in the final flow through of the immunoaffinity column.

To identify the subunit content of the immunopurified α6 subtype, we used immunoprecipitation to analyze the receptor eluted from the affinity column by the corresponding α6 peptides (Table 3). The β4 Abs immunoprecipitated almost all of the [^{3}H]Epi- labeled α6-receptors, thus indicating that almost all of the α6 receptors contain the β4 subunit; half were immunoprecipitated by anti-β3 Abs, 42% by anti-α3 Abs, and 7.5% by anti-β2 Abs. Because the anti-α4 and α5 Abs were able to immunoprecipitate these receptors only to a very limited extent, we think that these subunits are not coassembled with the α6 and β4 subunits.

The subunit composition of the purified α6 receptors was also analyzed on Western blots using the same panel of Abs as that used for the immunoprecipitation experiments (see Fig. 2). Both anti-α6 Abs recognized a peptide of molecular mass 57.1 ± 0.9 kDa (anti-α6 COOH lane 5 and anti-α6 CYT lane 6), the anti-β4 Abs recognized a single band of molecular mass 52.3 ± 0.5 kDa (anti-β4 COOH lane 11 and anti-β4 CYT lane 12), the anti-β2 COOH recognized a faint band of 54 kDa (lanes 8), and the anti-α3 recognized a peptide of molecular mass 56.3 ± 0.3 kDa (anti-α3 COOH lane 1 and anti-α3 CYT lane 2). The anti-β3 Abs directed against the COOH peptide did not recognize any peptide on the purified receptors, whereas the anti-β3 CYT recognized a band of molecular mass 55 ± 1 kDa (lane 10) that was also recognized by a serum obtained from rabbit immunized with the fusion protein containing the S105-I180 sequence of the extracellular β3 chick subunit (lane 9). We also tested the purified α6 receptors for the possible presence of α4 (lane 3), α5 (lane 4), and α7 (lane 7) but could not detect any labeling using subunit specific Abs. This absence of recognition is due to a

TABLE 2

Percentage of immunoprecipitation in COL and retina extracts of [^{3}H] Epi-labeled receptors by antisubunit-specific Abs and mAbs

Immunoprecipitation was carried out as described in *Materials and Methods* using saturating concentrations (20–30 μg) of antisubunit Abs or mAbs. Results are expressed as a percentage of [^{3}H]Epi-labeled receptors, taking the amount of receptor present in the solution before immunoprecipitation as 100%. Percentage of immunoprecipitation was subtracted from value obtained in control samples containing an identical concentration of normal rabbit or rat IgG. Values are mean ± S.E.M. of three determinations.

Abs or mAbs	COL	Retina
Anti-α3-COOH	8 ± 2	31 ± 2
Anti-α3-CYT	6 ± 1	25 ± 2
mAb 313	15 ± 3	42 ± 1.3
Anti-α4-COOH	86 ± 2	45 ± 5
Anti-α4-CYT	45 ± 2	25 ± 2
mAb 299	80 ± 5	44 ± 4
Anti-α5-COOH	25 ± 0.9	18 ± 3
Anti-α5-CYT	20 ± 0.2	14 ± 2
mAb35	30 ± 1	11 ± 3
Anti-α6-COOH	5 ± 1	34 ± 3
Anti-α6-CYT	4 ± 0.5	35 ± 4
Anti-β2-COOH	93 ± 5	45 ± 3
Anti-β2-CYT	89 ± 2	30 ± 2
mAb 270	95 ± 5	ND
Anti-β3-COOH	0.6 ± 0.2	28 ± 6
Anti-β3-CYT	5 ± 0.4	32 ± 2
Anti-β4-COOH	11 ± 2	84.2 ± 1.5
Anti-β4-CYT	13 ± 0.5	78 ± 2.5

TABLE 3

Immunoprecipitation analysis of subunit content of purified α6-containing receptors

α6 Receptors were immunopurified on an affinity column with bound Abs directed against the α6-CYT peptide. α6 Receptors were eluted from affinity column using 100 μM of α6 CYT peptide used for Ab production. After extensive dialysis to remove the peptide, receptors was labeled with 2 nM [^{3}H]Epi and immunoprecipitation was performed as described in Table 2. Values are mean ± S.E.M. of four determinations.

Abs	[^{3}H]Epi-labeled α6 Receptors
	%
Anti-α3-COOH	43 ± 6
Anti-α3-CYT	41 ± 8
Anti-α4-COOH	0.7 ± 0.9
Anti-α4-CYT	1.2 ± 1.5
Anti-α5-COOH	1.3 ± 0.8
Anti-α5-CYT	2 ± 1.4
Anti-α6-COOH	93 ± 5
Anti-α7-CYT	0
Anti-β2-COOH	8 ± 1
Anti-β2-CYT	7 ± 1
Anti-β3-COOH	46 ± 1
Anti-β3-CYT	56 ± 3
Anti-β4-COOH	94 ± 4
Anti-β4-CYT	95 ± 8

lack of proteins for the α4 and α5 subunits since the same Abs were able to recognize the appropriate subunits in receptors purified from COL using anti-β2 Abs, and the anti-α7 Abs recognized the appropriate peptide in receptors purified by affinity on Sepharose-αBgtx (data not shown).

The molecular masses of the α6, α3, and β4 subunits, determined by Western blot are given as the mean ± S.E.M. obtained from three to four experiments, and corresponded to the expected sizes deduced from their cDNA sequences. The M_r of the β3 subunit was slightly higher, but this may be due to glycosylation of the subunit because it has two potential glycosylation sites.

Pharmacological Experiments on α6 Subtype. The pharmacological experiments were all carried out on receptor immobilized by the corresponding anti-α6 Cyt-specific Abs as described in *Materials and Methods*.

The α6 receptors bind [^{3}H]Epi with high affinity; the K_d value calculated from 10 separate experiments was 35 pM [percentage of coefficient of variation (CV) = 18%].

Figure 3 shows a typical saturation curve of the total and nonspecific binding of [^{3}H]Epi to the immunoimmobilized subtype. The interaction of [^{3}H]Epi with the α6-receptors was consistent with the presence of a single class of high-affinity binding sites, and the Scatchard plot of the saturation curve also shown in Fig. 3 indicates the presence of a single class of high- affinity sites in these receptors.

The pharmacological profile of the α6 receptors was further characterized by testing the relative efficacies by which various cholinergic agonists and antagonists inhibit the binding of 0.1 mM [^{3}H]Epi at equilibrium.

Figure 4 shows the inhibition curves of cholinergic agonists (Fig. 4A) and antagonists (Fig. 4B) for the binding of [^{3}H]Epi to the immunoimmobilized subtype. The K_i values of the inhibition curves shown in Table 4 were obtained by simultaneously fitting the data from three to four separate experiments.

The relative efficacies of the agonists in the competition experiments were Epi ≫ Cyt > nicotine (Nic) > 1,1-dimethyl-4-phenylpiperazinium (DMPP) > ACh > Carb, and except for Carb, all of them had relatively low K_i values (in the low nanomolar range). The rank order of antagonist potencies was α-conotoxin MII (MII) > methyllycaconitine (MLA) > dihydro-β-erythroidine (DHβE) > MG624 > *d*-tubocurarine (*d*-TC) > decamethonium > hexamethonium. We found that the toxin MII, a compound described as a antagonist of the rat α3β2 subtype, is the most potent drug (K_i = 66 nM) in competing for α6 receptors, and has an affinity that is respectively 20 and 40 times higher than that of MLA (K_i 1.35 μM) and DHβE (K_i 2.8 μM).

To exclude possible interference by the immunoimmobilizating Abs on the pharmacology of the α6 receptors, we also tested the binding of [^{3}H]Epi and ACh in receptors immunoimmobilized on the anti-α6 COOH Abs. The results were qualitatively the same with a K_d of 30 pM (CV = 15%) for [^{3}H]Epi and a K_i of 80 nM (CV = 20%) for ACh.

Functional Reconstitution in Lipid Bilayers. To study the biophysical properties of the α6 receptors, the immunopurified receptors were reconstituted in lipid bilayers and their properties studied after agonist activation. In our experiments, traces with more than one channels were rare and are disregarded in the analysis.

Figure 5A shows the single-channel currents recorded from a planar lipid bilayer containing α6 receptor activated by 10 μM ACh. Figure 5B shows an amplitude histogram derived from the recording shown in Fig. 5A and Fig. 5C the distribution of the open- and closed-state lifetime evaluations. The open-channel lifetime followed a single exponential distribution of the channel with a mean open lifetime of 1.9 ms, whereas the closed time followed a double exponential distribution with a mean closed lifetime of 0.9 ms within bursts and 9 ms between bursts. This channel had a conductance of about 50 picosiemens and, in addition to the main open-state channel, also showed some other conductances, although their frequency of occurrence was very low.

Figure 6A shows traces of the same channel activated at increasing concentrations of ACh and Fig. 6B the overall open state probability of the channel as a function of the log

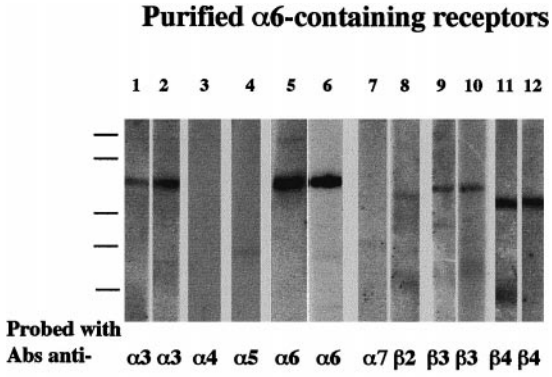

Fig. 2. Western blot analysis of the immunopurified α6 receptors. Retina extract was incubated three times with anti-α6 CYT Abs bound to Sepharose 4B, and the bound receptors eluted with 0.2 M glycine or the α5 CYT peptide. Eluted receptors were concentrated and separated on 9% acrylamide SDS gels, electrotransferred to nitrocellulose, and then probed with 5 to 10 μg/ml of anti-α3 COOH (lane 1), anti-α3 CYT (lane 2), anti-α4 COOH (lane 3), anti-α5 COOH (lane 4), anti-α6 COOH (lane 5), anti-α6 CYT (lane 6), anti-α7 CYT (lane 7), anti-β2 CYT (lane 8), anti-β3 fusion protein (lane 9), anti-β3 CYT (lane 10), anti-β4 COOH (lane 11), and anti-β4 CYT (lane 12). The bound Abs were revealed by means of ^{125}I-labeled protein A. Molecular mass markers (top to bottom) are 97, 66, 45, 31, and 21 kDa.

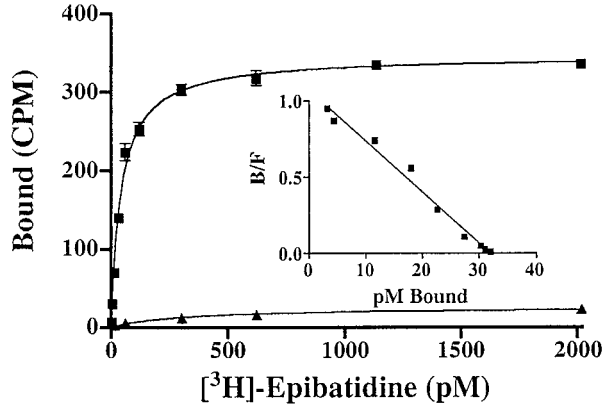

Fig. 3. Saturation curve of [^{3}H]Epi binding to immunoimmobilized α6 receptors, and its Scatchard analysis (insert). For the total binding the immunoimmobilized receptors were incubated overnight at 4°C with the indicated concentrations of [^{3}H]Epi and for nonspecific binding also in the presence of 100 nM Epi. The total (■) and nonspecific binding (▲) shown is that obtained from a representative experiment; the K_d value of 35 pM (c.v. = 18%) was calculated by simultaneously fitting 10 separate experiments. Scatchard plot of the saturation curve shows the presence of a single class of high-affinity sites.

of the ACh concentration. These experiments were performed six times with α6 receptors obtained from three separated immunopurifications.

The Hill coefficient calculated from the plot of the integral P_o as a function of agonist concentration was 1.8, thus indicating the cooperative activation of the channel by two or more agonist molecules. The ACh EC_{50} value determined by plotting the integral P_o as a function of agonist concentrations was 100 μM; the EC_{50} of the other nicotinic agonists tested Epi, Nic, and Carb were, respectively, 1.2 ± 0.5, 9.8 ± 0.7, and 965 ± 50 μM (mean ± SEM of 3–4 determinations). All of the α6 channels activated by nicotinic agonists were blocked by the nicotinic antagonist *d*-TC at 100 μM.

Figure 7 shows the current-voltage relationship for the α6 subtype, which can be fitted by a straight line with a main conductance of 48 pS. The P_o of the channels was found to be voltage dependent (Fig. 7B) and was tested in 10 separate experiments.

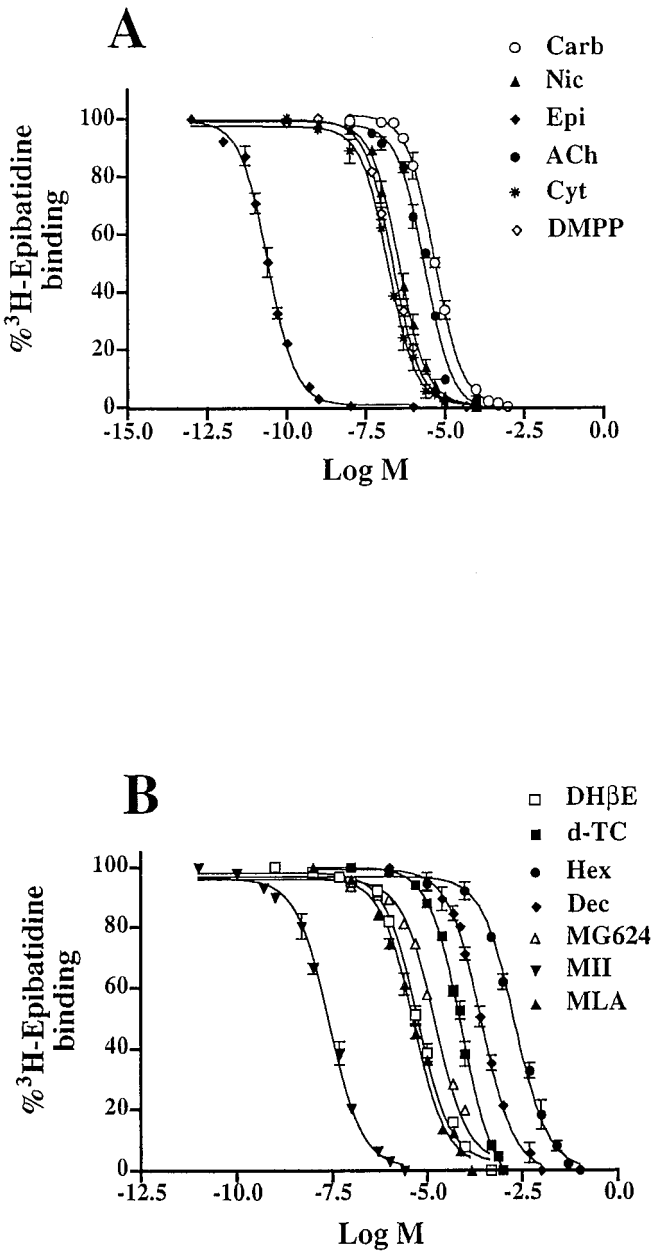

Fig. 4. Inhibition by nicotinic agonists (A) and antagonists (B) of [³H]Epi binding to immunoimmobilized α6 receptors. α6 Receptors immunoimmobilized on the anti-α6 Cys Abs (as described in *Materials and Methods*) were preincubated for 30 min at 20°C with the indicated concentrations of nicotinic ligands; [³H]Epi at a final concentration of 100 pM was then added, and the mixture left overnight at 4°C. The curves were obtained by fitting 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 [³H]Epi-specific binding to the receptors (considered as 100%).

Discussion

The only previously available data about the α6 subunit were its cloning, its mRNA distribution in rat brain and some areas of the chick central nervous system, and the electrophysiological properties of α6 receptors heterologously expressed in oocytes and mammalian cells; no data were available concerning the structure and function of native α6-containing receptors. However, it has recently been shown that the functional and pharmacological properties of heterologous subtypes may be influenced by the kind of cell expressing them, which means that the results need to be interpreted very carefully (Fucile et al., 1997; Lewis et al., 1997).

One approach to identifying the subunit composition of native receptors is to purify them using subunit-specific Abs. The reliability of the present work depends on the specificity of the Abs, which is why we raised them against two distinct epitopes of the same subunit and tested them all in immunoprecipitation experiments using detergent-solubilized receptors of two neuronal tissues that are known to have different nAChR subtype expressions.

Although there was a difference in the absolute percentages of receptors immunoprecipitated by the anti-α3 and anti-α6 Abs in COL and retina, the percentage of receptors immunoprecipitated by the Abs were similar in the individual tissues (see Table 2). In order to exclude any cross-reactivity between the anti-α3 and anti-α6 Abs, the specificity of this Abs was confirmed on human cell lines expressing the chick α3β4 or α6β4 subtypes.

Using these Abs, in agreement with other published data (Morris et al., 1990; Whiting et al., 1991; Vernallis et al., 1993; Conroy et al., 1995; Conroy and Berg, 1997), we found

TABLE 4

Pharmacological profile of α6 receptors

K_d and K_i values were derived from curves of [³H]-Epi saturation and competition binding to α6-immunoimmobilized receptors. Curves obtained from three separate experiments were fitted using a nonlinear least-squares analysis program and the *F* test (Munson and Rodboard). Numbers in parentheses represent percentage of CV.

Ligand	K_i
	nM
Epi	0.020 (32)
Cy	11 (36)
Nic	20 (31)
DMPP	31 (37)
ACh	76 (26)
Carb	975 (29)
MII	66 (24)
MLA	1350 (25)
DHBE	2800 (13)
MG624	4520 (26)
d-TC	7700 (18)
Decamethonium	35900 (16)
Hexamethonium	349000 (18)
[³H]Epi	K_d = 35 pM (18)

that almost all of the receptors in chick brain contain the α4 and β2 subunits, and only a minority contain the α5, α3 and β4 subunits.

Fucile et al. (1998) and Hernandez et al. (1995) reported that there is a high level of α6 and β3 subunit mRNA in chick retina, whereas these mRNAs are undetectable in the optic tectum. In agreement with their results, we found that both COOH and CYT Abs were able to immunoprecipitate a consistent amount of [^{3}H]Epi-labeled receptors in retina but not in the chick optic tectum (less than 6%), and so we used the former for the purification of the native receptor containing the α6 subunit.

Immunoprecipitation and Western blot analyses of the purified α6-containing receptors showed that almost all of them also contained the β4 subunit, one-half contained the β3 subunit, 42% the α3 subunit, and 7.5% the β2 subunit. These results show that our purified receptors are a mixture of different populations of α6-containing receptors (surely α6β4; probably α6β4β3, α3α6β4, and/or α3α6β3β4; and possibly a minor subpopulation containing the β2 subunit) but we do not know whether the α3 subunit coexists with the β3 and/or β2 subunit. Binding analyses of the immunoimmobilized α6 receptors showed the presence of a single population

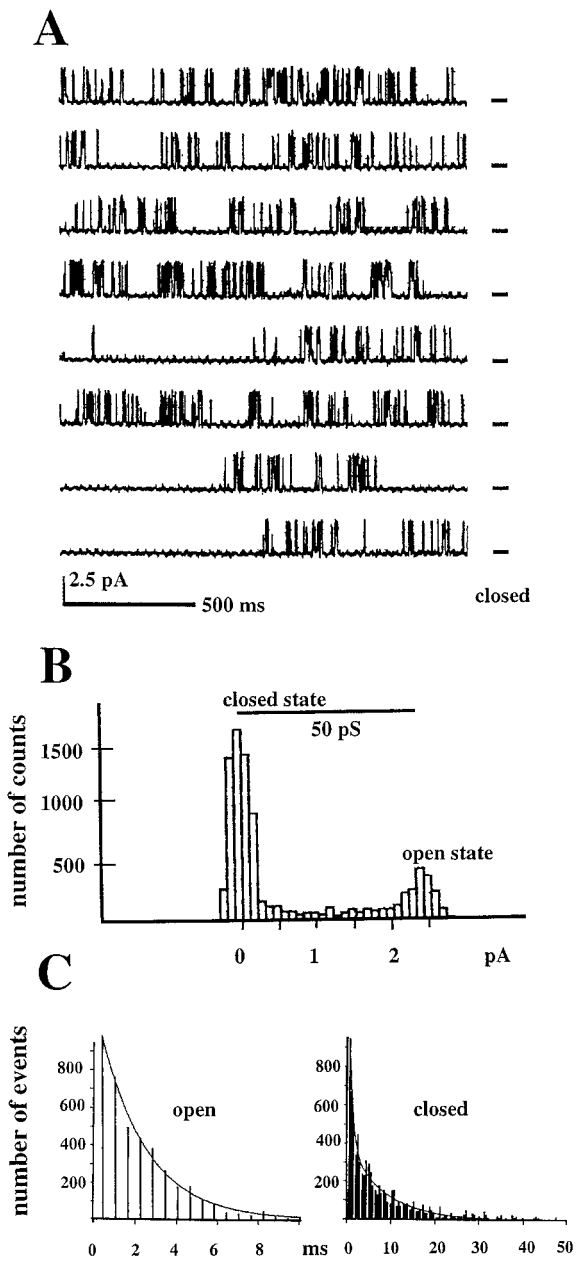

Fig. 5. A, traces of the single α6 receptor channel activated by ACh 10 μM. Applied membrane voltage was 50 mV. Traces were digitized at a sampling rate of 2000 Hz. B, amplitude histograms of traces shown in A (measured from a 5-s trace at 2000 samples/s) had well-defined closed and open states, with the open state having a major conductance of 48 pS. Bar represents 50 pS. y-Axis is arbitrarily scaled in number of counts. C, histograms of the open- and closed-state lifetimes of the traces shown in part A. Open states show a single and closed states a double exponential distribution. Mean open lifetime of the α6 channel was 1.9 ms; mean closed-state lifetime was 0.9 within bursts and 9 ms between bursts. Both histograms were constructed from a total of 3500 events

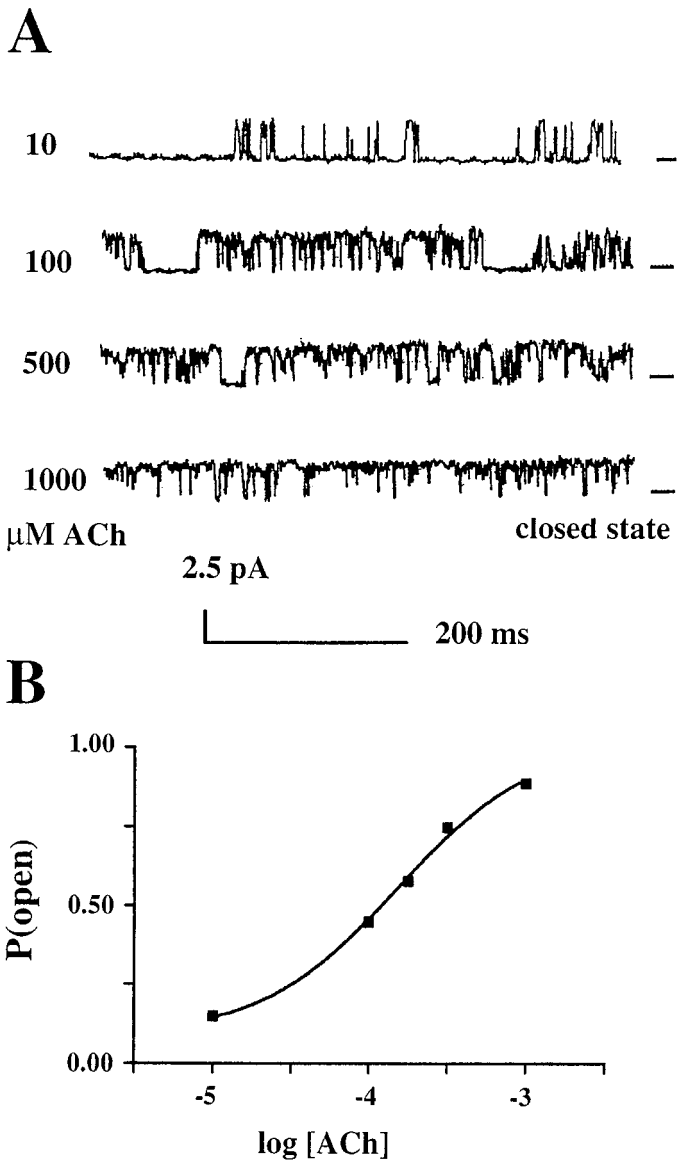

Fig. 6. Agonist concentration dependence of the P_o of one α6 receptor channel reconstituted in a planar lipid bilayer. A, channel was tested at different ACh concentrations; the applied membrane voltage was 50 mV. B, probability of the α6 receptor channel being in the open state (taken from all-point amplitude histograms) is shown as a function of the ACh concentrations. All of the values were measured at a membrane potential of 50 mV; each curve represents the data from one bilayer and one channel. Other channels (n = 10) gave comparable results. Hill slope of the fitted curve was 1.8 and the EC_{50} value was 100 μM.

of high-affinity [^{3}H]Epi binding sites, and the competition experiments with nicotinic agonists and antagonists did not reveal any binding site heterogeneity. The electrophysiological studies showed that the reconstituted receptors form a channel with a main conductance state of 48 pS, with other conductance levels being much less frequent. The homogeneity in the pharmacological and biophysical properties of α6 receptors may be surprising, but could be due to the fact that the presence of the β3 or/and α3 subunits does not modify their affinity for nicotinic drugs but regulates some other channel properties and/or confers other properties unrelated to channel function (e.g., localization), as has been recently demonstrated for the intracellular loop of the α3 subunit (Williams et al., 1998). Another possibility is that the affinities in the different subtypes are so close that we cannot discriminate them by binding experiments. The fact that the presence of the β3 subunits does not seem to change the affinity for nicotinic ligands is in agreement with the data reported by Groot-Kormelink et al. (1998), who found no detectable shift in the ACh concentration-response curve in human α3β4 receptors expressed in oocytes alone or together with the β3 subunit. However, it seems that the presence of the α3 subunit (together with the α6 and β4 subunits) in human transfected cells leads to a 3-fold decrease in the affinity for ACh (Fucile et al., 1998).

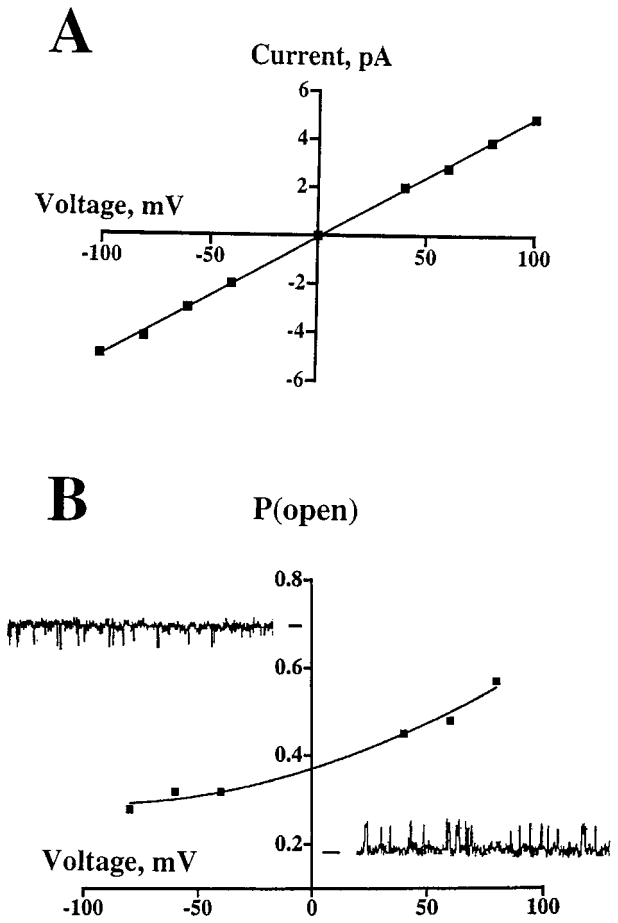

Fig. 7. A, I-V curve of the main conductance level of the α6 receptor channel at 100 μM ACh. B, P_o of the α6 receptor channel shown in A plotted as a function of voltage. Inserts show the traces at −60 mV and +60 mV.

The order of potency of nicotinic agonists is Epi > Cyt > Nic > DMPP > ACh > Carb, which, at least for Epi > Nic > ACh > Carb, is in line with our findings in reconstituted receptors, and very similar to the rank order in oocyte-expressed chick-human α6β4 receptors.

The pharmacological profile of the rat forebrain α4β2 subtype has recently been compared with that of the human α3β4 subtype expressed in human embryonic kidney cells (Xiao et al., 1998). These two subtypes have a similar rank order for agonists but markedly different K_i values: the K_i values that we determined for the α6 receptors are closer to those of the α4β2 than to those of the α3β4 subtype.

The most interesting finding in the antagonist pharmacological profile is the extremely high affinity for the toxin MII (K_i = 66 nM). This toxin is very selective for the oocyte-expressed rat α3β2 subtype (IC_{50} 3.5 nM; Cartier et al., 1996), and further work is necessary to establish whether this high affinity for α6 receptors is due to species and/or subtype differences. Interestingly, MLA inhibited [^{3}H]Epi binding with a K_i of 1.3 μM, whereas it is known to inhibit the binding to the chick α4β2 subtype with a much higher K_i (>50 μM; Maggi et al., 1999); this low K_i is in agreement with the finding of Fucile et al. (1998), who found that 10 μM MLA blocks induced responses in the oocyte-expressed α6β4 subtype. Furthermore, the K_i of 2.8 μM of DHβE (a drug that acts on multiple subtypes and discriminates their different subunit composition), is intermediate between its very low K_i for the α4β2 rat subtype (29 nM) and its very high K_i for the rat α3β4 subtype (>200 μM) (Cartier et al., 1996).

Finally, MG624 (a selective antagonist for the chick α7 homomeric receptor) binds the chick α7 subtype with a K_i of 106 nM (Gotti et al., 1998; Maggi et al., 1999), but the K_i of its binding to native α6 receptors is 40 times higher.

In conclusion, our binding data show that the presence of the α6 subunit gives AChRs a pharmacological profile that is different from that of both the homomeric subtype and the heteromeric α4β2 and α3β4 subtypes, and also confers a high affinity for nicotinic agonists. When reconstituted in lipid bilayers, the purified α6 receptors formed functional channels that were activated by agonists in a dose-dependent manner and blocked by *d*-TC. The main conductance of these channels is 48 pS, although channels with other conductances are less frequently present. The single channel has an ohmic behavior, but its P_o is voltage dependent. Depending on physiological conditions and voltage sign definition, this may account for some channel rectification in agreement with the results of as Gerzanich et al. (1997), who found a strong inward current rectification of the α6β4 subtype expressed in oocytes at both positive and negative potentials. The EC_{50} values of the agonists on the reconstituted receptors were 1000 times greater than their K_i values determined in our binding experiments. This discrepancy could be due to the fact that binding studies are performed on desensitized receptors that have a high affinity for nicotinic agonists, or that reconstituted receptors do not mimic all of the properties of their "in situ" native receptors. The fact that the EC_{50} value (100 μM) of ACh in reconstituted receptors is very similar to those obtained for the chick α6β4 subtype in transfected cells (EC_{50} 105 μM), and lower but always in the same range as that obtained with the α6β4α3 subtypes (EC_{50} 204 μM) (Fucile et al., 1998), strongly supports the first hypothesis.

Like other neuronal subtypes purified and reconstituted using the same technique (Gotti et al., 1994, 1997), the α6 receptors do not show any desensitization at high agonist concentrations. We believe that this may be due to: 1) a lack of certain lipids in lipid bilayers that could be important for receptor activation and fast desensitization, as has recently been demonstrated in the case of cholesterol and torpedo receptor (Rankin et al., 1997); 2) the purification and/or reconstitution procedures may favor an open conformation of the desensitized receptor, as has been shown to occur in the homomeric α7 channel, in which the mutation of a single site in the M2 region (Leu 247 to Thr) yields an additional higher conductance channel (80 pS) that desensitizes very slowly (Revah et al., 1991); and/or 3) the removal of certain regulatory factors or peptides that are possibly present in native membrane that may play a role in channel properties.

Another point that emerges from our data is that the α6β4-containing receptors are heterogeneous and can contain other α and β subunits, whereas our pharmacological and functional studies revealed only a single class of receptors. This may be explained by one of two different hypotheses: 1) the α6β4 subunits are the major determinants of the characteristics of the binding sites and channel properties, or 2) the other receptor subtypes are a minority and their properties cannot be detected by our assays.

Acknowledgments

We thank Dr. Ballivet and Dr. McIntosh for their generous gifts of β3 fusion protein and toxin MII and Mr. Kevin Smart and Ms. Ida Ruffoni for their aid with the manuscript.

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