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Novel Subpopulation of Neuronal Acetylcholine Receptors Among Those Binding α-Bungarotoxin

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SUMMARY

Neuronal acetylcholine receptors (AChRs) that bind α-bungarotoxin (αBgt) (αBgt-AChRs) have previously been found to contain at least one of the α7–α9 gene products. No other gene products of the 11 neuronal AChR genes cloned to date from rat and/or chick have been identified in such receptors. Chick ciliary ganglia have about 20 fmol of αBgt-AChRs that contain α7 subunits and 5 fmol of synaptic-type AChRs that bind the monoclonal antibody (mAb) 35 and collectively contain α3, β4, α5, and, to a lesser extent, β2 subunits. Using a sensitive solid-phase immunoprecipitation assay, we show here that ciliary ganglia have about 1 fmol of novel putative AChRs that bind both αBgt and mAb 35 but appear to lack all of the known neuronal AChR gene products in ciliary ganglia, including α3, α5, α7, β2, and β4. The putative receptors are also unlikely to contain either α8 or α9 gene products, because of the known expression patterns of these gene products. Nonetheless, the component sediments at 10 S, as expected for neuronal AChRs, and has a nicotinic pharmacology similar but not identical to that of α7-containing αBgt-AChRs. The AChR α1 gene product expressed in muscle is known to bind both αBgt and mAb 35, and we show here that ciliary ganglia contain small amounts of α1 transcript. The putative ciliary ganglion AChR defined by joint αBgt and mAb 35 binding, however, does not appear to contain α1 subunits. A similar component binding both mAb 35 and αBgt can be detected in sympathetic ganglia and dorsal root ganglia but not in brain, spinal cord, or retina. The developmental time course of the component in ciliary ganglia is comparable to that of the α7-containing αBgt-AChRs. If the component is a functional AChR on ciliary ganglion neurons, as seems likely, it would represent the fourth AChR subtype produced by this population of cells. Our inability to identify subunits comprising the putative receptors raises the possibility that additional AChR genes remain to be cloned.

Neuronal nicotinic αBgt-AChRs are widely distributed in both the central and peripheral nervous systems (for reviews, see Refs. 1 and 2). Despite their abundance, only recently has a physiological response been demonstrated for a native receptor of this class. Rapid application of agonist activates αBgt-AChRs on chick ciliary ganglion neurons, showing the receptors to be ligand-gated ion channels that are cation selective, prefer nicotine over ACh, and rapidly desensitize (3). The receptors are efficient at elevating intracellular calcium levels (4) and can influence calcium-dependent events in neurons, such as the extension or retraction of neurites (5). The physiological significance of neuronal αBgt-AChRs *in vivo*, however, remains unclear because, at least on ciliary ganglion neurons, the receptors are found primarily in nonsynaptic regions (6, 7).

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The combinations of gene products making up neuronal αBgt-AChRs remain a matter of conjecture. Eleven neuronal AChR genes have been cloned to date. Eight (α2–α9) encode subunits thought to bind ligand directly, whereas three (β2–β4) encode subunits thought to be more structural in purpose, although both classes of gene products can influence the pharmacology of AChRs (2). Expression of the α7 gene product alone in *Xenopus* oocytes produces functional receptors that can be blocked by αBgt (8). This has led to speculation that native αBgt-AChRs may be homomeric. Indeed, subunit analysis of neuronal αBgt-AChRs has identified a species containing only α7 subunits of the 11 known neuronal AChR gene products; others contain either α8, α7 plus α8, or α9 (9–12). Pharmacological comparisons of α7-containing homomers and native αBgt-AChRs, however, have revealed subtle differences, suggesting that the native receptors may be heteromeric (13, 14). In addition, purification of neuronal αBgt-AChRs, followed by SDS-polyacrylamide gel electro-

ABBREVIATIONS: αBgt-AChR, acetylcholine receptor that binds α-bungarotoxin but not monoclonal antibody 35; AChR, acetylcholine receptor; αBgt, α-bungarotoxin; αT/35-AChR, putative acetylcholine receptor that binds α-bungarotoxin and monoclonal antibody 35; mAb, monoclonal antibody; mAb 35-AChR, acetylcholine receptor that binds monoclonal antibody 35 but not α-bungarotoxin; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; ACh, acetylcholine; E8–19, embryonic days 8–19; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

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phoresis analysis, has revealed multiple components as candidates for receptor subunits (15–17), with only a subset of the components appearing to be derived from either α7 or α8 gene products (17).

On chick ciliary ganglion neurons the extrasynaptic αBgt-AChRs vastly outnumber the AChRs responsible for mediating synaptic transmission. These latter receptors are mAb 35-AChRs. Collectively, mAb 35-AChRs contain the α3, β4, and α5 gene products but not α7 gene products, whereas the reverse is true for αBgt-AChRs (11). About one fifth of the mAb 35-AChRs contain β2 subunits but none of the αBgt-AChRs do so (18). We have used a sensitive solid-phase immunodetection assay to examine αBgt-AChRs and have discovered a novel minor population of receptors that bind both αBgt and mAb 35. The putative receptors do not appear to contain any of the known neuronal AChR gene products or the one muscle AChR gene product that could account simultaneously for the αBgt and mAb 35 binding properties. The results suggest that additional AChR genes remain to be cloned and that individual neurons may have as many as four classes of AChRs with different subunit compositions.

Materials and Methods

Tissue extracts. Ciliary ganglia and pectoral muscles were dissected from chick embryos on E8, E11, E14–15, and E17–19. Whole brains, cerebella, retinae, and livers were dissected from E17–18 embryos. Spinal cords, sympathetic ganglia, and dorsal root ganglia were dissected from E14–15 embryos. All tissues used for protein extraction were frozen at −70° until used.

Tissues were ground in a glass homogenizer (Wheaton) and extracted with a buffer containing 50 mM sodium phosphate, pH 7.4, 0.4 mM iodoacetamide, 5 mM benzamidine, 5 μg/ml phosphoramidon, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml leupeptin, 20 μg/ml pepstatin A, 5 mM EDTA, 5 mM EGTA, 2 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, and either 1 or 2% Triton X-100 (Pierce). For ciliary ganglia, brains, retinae, and cerebella, extraction was performed in 1% Triton X-100, and the homogenate was immediately centrifuged in a microfuge to remove insoluble components. Spinal cords and pectoral muscles were homogenized and then solubilized in 2% Triton X-100 for at least 2 hr at 4° before centrifugation. Protein concentrations were measured using a Bio-Rad protein assay.

Solid-phase immunoprecipitation assays. Solid-phase immunoprecipitation assays to quantify individual receptor subtypes in cell extracts were performed as described previously (19). Briefly, microtiter wells (Immulon-2 Removawells; Dynatech Laboratories) were coated overnight with either rabbit anti-rat or anti-mouse IgG (Jackson ImmunoResearch), at 20 μg/ml, in PBS (10 mM sodium phosphate, pH 7.4, 150 mM sodium chloride) containing 0.02% sodium azide. The wells were then rinsed three times and incubated with the desired primary mAb in PBS-azide either for 6 hr at 37° or overnight at 4°, followed by additional rinsing. Extract samples were then incubated in the wells overnight at 4°. In some experiments, sequential incubations were performed on the same extracts by recovering the extract samples after the first incubation with tethered mAbs and then subjecting them to a second incubation in new wells with antibody. Sequential incubations were used to assess the efficiency of the solid-phase immunoprecipitation assay for each of the several receptor subtypes of interest. Primary mAbs in these experiments were used at a concentration of about 1.3 μg/well, except for 318 and 319, which were present at about 0.13 μg/well each, and 270, which was present at about 0.25 μg/well.

To determine the amount of bound receptor, the wells were rinsed three times and then incubated for 2 hr at 37° either with 10 nM ^{125}I-αBgt in PBS containing 0.5% Triton X-100 plus 1% (v/v) fetal bovine serum or with 5 nM ^{125}I-mAb 35 in PBS- Triton X-100 containing both 2% (v/v) normal mouse serum and normal rat serum. (For ^{125}I-mAb 35 incubations, the wells were treated with vehicle for 30–60 min at 37° before addition of labeled antibody.) After an additional four rinses with PBS-Triton X-100, the wells were quantified for bound radioactivity using a γ counter. Nonspecific binding was assessed either by including 1 μM unlabeled αBgt or 5 μM unlabeled mAb 35 in the binding reactions or by omitting extracts from the binding reaction. Results are expressed as the mean ± standard error values obtained for the indicated number of trials and represent specific binding, as determined by subtracting nonspecific binding from total binding.

When competition binding experiments were performed with ^{125}I-αBgt, the incubations included the indicated compounds and were carried out for 24 hr at 4°, to ensure that equilibrium was reached. Plots of competition curves were generated and fit using InPlot (GraphPad Software). K_i values were calculated as described previously (20).

Affinity purifications. Receptors were partially purified using either toxins or mAbs coupled to Actigel resin, as described previously (21). The αBgt resin was prepared by coupling αBgt (at 5–6 mg/ml) to Actigel (Sterogene) according to the manufacturer's directions. αBgt-Actigel beads were washed with PBS-Triton X-100 containing 1 M sodium chloride, followed by three washes with PBS-Triton X-100 before use. Extracts were incubated with coupled beads overnight at 4° on a rotating platform. Binding of ^{125}I-αBgt to bound receptors was carried out as described above for wells, except that the incubations were at room temperature instead of 37°. For immunoblot analyses, receptors were eluted from mAb-Actigel as described (11) and from αBgt-Actigel using SDS sample buffer. In the latter case, the efficiency of the elution was assessed by adsorbing *Torpedo* electric organ AChRs onto the resin, eluting the receptors, and quantifying the amount recovered in immunoblots probed with mAb 61, which recognizes the α1 gene product (22). At least three quarters of the α1 protein present in the original *Torpedo* extract were recovered after adsorption and elution from the resin, verifying the efficiency of the recovery.

Immunoblot analysis. Ciliary ganglion AChRs immunopurified from detergent extracts were analyzed on immunoblots as described (19), with the following modifications. Samples to be analyzed by immunoblotting for either α1 or α5 protein were not heated after solubilization in SDS; α7 samples were heated to 100° for 2 min before being loaded onto the gel because the heating induced aggregation of α7 protein, which increased the sensitivity of the antibodies for unknown reasons. Proteins were blotted to either nitrocellulose (Hoeffer) or polyvinylidene difluoride (Immobilon-P; Millipore) membranes. The blots were blocked with 3% (w/v) nonfat dry milk (Carnation) in PBS containing 0.05% Tween-20 and 0.02% sodium azide, incubated for a period of overnight to 3 days at 4° with mAbs diluted in the same solution, washed in PBS-Tween-20, and then incubated for 2 hr at room temperature with horseradish peroxidase coupled to goat anti-rat IgG (Jackson ImmunoResearch), in 3% milk in PBS-Tween-20, to detect bound mAbs. Signals were visualized by enhanced chemiluminescence (Amersham or NEN). Dilutions of mAbs from concentrated stocks were as follows: mAb 268, 1/1000; mAb 61, 1/550; mAb 318, 1/100. Molecular mass markers for the blots (Bio-Rad, low range) included phosphorylase B (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), and carbonic anhydrase (31 kDa). In some cases, the same molecular mass markers were used in a prestained form (Bio-Rad, low range), with apparent molecular masses of 140 kDa, 87 kDa, 48 kDa, and 33 kDa, respectively.

Sucrose density gradients. Extracts were fractionated by sucrose gradient sedimentation as described previously (19), with the following changes: 0.20-ml aliquots (40 ganglia) were loaded onto 5-ml gradients and centrifuged for 70 min in a VTi 65.2 rotor (Beckman) at 65,000 rpm at 4°. The collected fractions were analyzed in solid-phase immunoprecipitation assays.

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RNase protection assays. RNase protection experiments were carried out as described previously (23). RNA samples were prepared in the following manner. E11 and E15 ciliary ganglia, E15 pectoral muscle, and E17–18 liver were dissected and frozen immediately in liquid nitrogen. Total RNA was extracted with an acid guanidinium thiocyanate-phenol-chloroform mixture as described previously (24). RNA was quantified by measuring A_{260}. For the protection experiments, a ^{32}P-labeled riboprobe for α1 was generated by run-off transcription using methods and DNA constructs described previously (19, 23). The sizes of the full length and the expected protected length of the α1 probe were 408 and 224 bases, respectively.

Materials. White Leghorn embryonated chick eggs were obtained locally and maintained at 39° in a humidified incubator. Methyllycaconitine was the kind gift of Dr. M. H. Benn (University of Calgary, Calgary, Canada). αBgt was purified from *Bungarus multicinctus* venom as described previously (25) and was radioiodinated to a specific activity of 3–7 × 10^{17} cpm/mol, using chloramine T. mAbs were purified or obtained as concentrated stocks or ascites fluids as described previously (11, 21). Purified mAb 61, which recognizes the α1 subunit of muscle and electric organ AChRs (22), was generously provided by Dr. Jon Lindstrom (University of Pennsylvania). *Torpedo* electric organ extracts were prepared as described previously (26), using tissue kindly supplied by Dr. Palmer Taylor (University of California, San Diego). ACh was purchased from Research Biochemicals. All other materials were purchased from Sigma, unless otherwise indicated.

Results

Neuronal components that bind both αBgt and mAb 35. A large population of αBgt-AChRs previously identified in chick ciliary ganglia contain the α7 gene product and do not bind mAb 35 (11). Using a sensitive solid-phase immunoprecipitation assay, we now find evidence for an additional population of receptors that do bind both αBgt and mAb 35 and are not recognized by anti-α7 subunit mAbs.

Extracts prepared from E17–19 ciliary ganglia were incubated in a solid-phase assay using mAb 35 as the tethering antibody to retain receptor. About 1 fmol of ^{125}I-αBgt binding sites/ganglion was recovered in the assay (Table 1). The binding was specific, in that it was blocked when excess unlabeled αBgt was included in the incubation. Retention of the binding component by mAb 35 was specific, because it could be duplicated by mAb 210, which competes with mAb 35 for binding in those cases where the two have been compared (21, 22), and because no ^{125}I-αBgt binding was observed when several other anti-AChR mAbs were substituted for mAb 35. These latter included mAb A3–1, which is specific for the α3 gene product, mAb B4–1, which is specific for the β4 gene product, mAb 270, which is specific for the β2 gene product, and mAb 308, which is specific for the α8 gene product (Table 1) (9, 11). In addition to demonstrating specificity, the inability of anti-α3, anti-β4, anti-β2, and anti-α8 subunit mAbs to retain αBgt binding indicates that these subunits are unlikely to be constituents of the component that binds both mAb 35 and αBgt.

Most interesting were solid-phase assays with the α7-specific mAbs 318 and 319. The mAbs tethered a large number of ^{125}I-αBgt binding sites, as expected from previous studies showing that ciliary ganglion αBgt-AChRs contain α7 subunits. Immunodepletions with mAbs 318 and 319 were efficient, because only a small fraction of the ^{125}I-αBgt binding sites remained in the recovered supernatants (Fig. 1). Despite this efficiency, mAbs 318 and 319 were unable to immunodeplete the component capable of binding both αBgt and mAb 35, indicating that the component is unlikely to contain α7 subunits. It is also clear that the ^{125}I-αBgt binding sites associated with material retained by mAb 35 in the solid-phase assay do not represent weak affinity of mAb 35 for α7-containing αBgt-AChRs. Immunodepletion with mAb 35 removed nearly all of the ^{125}I-αBgt binding sites that could be tethered in the solid-phase assay, without removing any of the sites tethered by mAbs 318 and 319 (Fig. 1).

TABLE 1

Ciliary ganglion components that bind both αBgt and mAb 35

Ciliary ganglion extracts from E17–18 chick embryos were incubated in the solid-phase immunoprecipitation assay, with the indicated mAbs being used to tether receptors. Antibody specificities are indicated in parentheses (MIR, "main immunogenic region") (22). ^{125}I-αBgt (10 nM) was used to quantify the number of αBgt binding sites associated with the material retained by the mAbs. Specific binding was determined by including an excess of unlabeled αBgt (1 μM) with the ^{125}I-αBgt and subtracting the values obtained from the total binding. Results are expressed as the mean ± standard error for *n* determinations. The results identify an αBgt-binding component that is recognized by the anti-main immunogenic region mAbs 35 and 210 but not by mAbs specific for α3, β4, β2, or α8 proteins. Positive controls confirmed that mAbs A3–1 and B4–1 immunoprecipitated mAb 35-AChRs (data not shown). The anti-α7 mAbs 318/319 immunoprecipitated a large number of ^{125}I-αBgt binding sites, representing previously identified ganglionic αBgt-AChRs.

Tethering mAb (specificity)	αBgt sites	*n*
	fmol/ganglion	
mAb 35 (α1/α5/MIR)	1.0 ± 0.1	35
mAb 210 (α1/α5/MIR)	1.0 ± 0.2	3
mAbs A3–1/B4–1 (α3/β4)	0.1 ± 0.1	3
mAb 270 (β2)	0.2 ± 0.1	5
mAb 308 (α8)	0.1 ± 0.1	3
mAbs 318/319 (α7)	21.6 ± 2.0	22

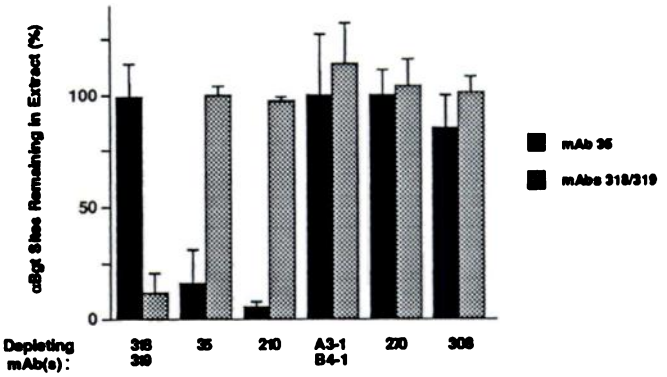

Fig. 1. Immunodepletions with subunit-specific mAbs followed by solid-phase assays, identifying a unique population of components that bind αBgt and mAb 35 but not mAbs specific for α3, α7, α8, β2, or β4 gene products. Extracts prepared from E17–18 ciliary ganglia were immunodepleted by incubation with the indicated mAbs [*Depleting mAb(s)*] and then measured for the number of remaining ^{125}I-αBgt binding sites that could be tethered either by mAb 35 (■) or mAbs 318/319 (▩) in the solid-phase immunoprecipitation assay. The results are shown as a percentage of the binding sites present in undepleted extracts and represent the mean ± standard error for three to 23 experiments. The 100% values were 1.0 ± 0.1 and 21.6 ± 2.0 fmol/ciliary ganglion equivalent for mAb 35- and mAb 318/319-tethered binding, respectively (Table 1). mAb 35 did not deplete the αBgt-binding components (αBgt-AChRs) recognized by mAbs 318/319, although it did deplete a component capable of binding αBgt. Conversely, mAbs 318/319 did not deplete the αBgt-binding components recognized by mAb 35, although they did deplete αBgt-AChRs. mAb 210 mimicked mAb 35, as expected (the two mAbs compete for binding to AChRs), whereas mAbs specific for α3, β4, β2, and α8 (mAbs A3–1, B4–1, 270, and 308, respectively) failed to deplete any αBgt-binding components.

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Neither class of ^{125}I-αBgt binding sites was depleted by mAb A3–1, B4–1, 270, or 308. Taken together, the solid-phase assays indicate that the component binding both αBgt and mAb 35 is a previously uncharacterized species.

Comparative pharmacology of αBgt-binding components. The pharmacological profile of the αBgt-binding component recognized by mAb 35 was determined for comparison with the more abundant α7-containing αBgt-AChRs in the ganglia. Competition binding studies with ^{125}I-αBgt and cholinergic ligands were carried out using the solid-phase assay. mAb 35 was used to tether the component binding both αBgt and mAb 35, whereas mAbs 318 and 319 were used to tether the α7-containing αBgt-AChRs. Scatchard analysis indicated that the former has a K_d for αBgt of about 1 nM, similar to that of αBgt-AChRs (Fig. 2A; Table 2). K_i values determined for a number of nicotinic cholinergic agonists and antagonists demonstrated that the two kinds of αBgt-binding components have very similar binding properties (Fig. 2B; Table 2). The primary difference was the 5–10-fold divergence between the two classes of αBgt-binding components in their affinities for ACh and nicotine. The αBgt-binding component recognized by mAb 35 clearly has a nicotinic pharmacology different in only minor respects from that of α7-containing αBgt-AChRs.

Size of the αBgt-binding component recognized by mAb 35. The size of the ciliary ganglion component binding both αBgt and mAb 35 was assessed by sucrose gradient sedimentation. Ciliary ganglion extracts were centrifuged through sucrose gradients (40 ganglion equivalents/tube), and the recovered fractions were assayed in the solid-phase assay for ^{125}I-αBgt binding, using either mAb 210 or mAbs 318 and 319 together as the tethering antibodies. Ciliary ganglion αBgt-AChRs, detected by retention with mAbs 318 and 319 in the solid phase, sedimented as a 10 S species, as reported previously (27). The component binding both αBgt and mAb 35, detected by retention with mAb 210 in the assay, also sedimented primarily as a 10 S species (Fig. 3). In addition, in some experiments as much as half of the material sedimented more slowly, spanning the 7–9 S region of the gradient. This more slowly sedimenting material may represent assembly intermediates detected in the assay. Nonetheless, the results demonstrate that a significant portion of the material binding both αBgt and mAb 35 has the size expected for a fully assembled neuronal AChR. For simplicity it is referred to below as the αT/35-AChR, although a function has yet to be identified for the component.

TABLE 2

Ligand binding properties of αBgt-binding components from chick ciliary ganglia

αBgt-binding components immunotethered in solid-phase assays with either mAb 35 (αT/35-AChRs) or mAbs 318/319 (αBgt-AChRs) were subjected to direct binding with ^{125}I-αBgt and competition binding with ^{125}I-αBgt and cholinergic ligands, as described for Fig. 2, to determine their pharmacological profiles. The values shown are K_d values for ^{125}I-αBgt and K_i values determined by the method of Cheng and Prusoff (20), for all other entries; they represent the mean ± standard error for the number of determinations indicated in parentheses. The two classes of αBgt-binding components have similar pharmacologies, except for their differences in affinity for ACh and nicotine.

Ligand	K_d or K_i for competition with ^{125}I-αBgt	
	αT/35-AChRs	αBgt-AChRs
^{125}I-αBgt	0.99 ± 0.10 nM (3)	1.28 ± 0.12 nM (3)
Nicotine	90 ± 18 nM (2)	488 ± 11 nM (2)
Cytisine	234 ± 90 nM (3)	318 ± 56 nM (3)
ACh	8.43 ± 4.85 μM (3)	70 ± 11 μM (3)
Methyllycaconitine	0.47 ± 0.20 nM (3)	0.51 ± 0.18 nM (3)
d-Tubocurarine	3.67 ± 1.20 μM (3)	6.06 ± 1.78 μM (4)
Gallamine	7.60 ± 5.19 μM (3)	6.26 ± 2.54 μM (3)
Strychnine	18.9 ± 8.60 μM (3)	4.54 ± 1.31 μM (3)
Decamethonium	198 ± 135 μM (2)	295 ± 79 μM (3)

Determination of the α1 mRNA in ciliary ganglia. The solid-phase immunoprecipitation experiments described above rendered unlikely the possibility that αT/35-AChRs contain either α7 or α8 subunits. Ciliary ganglia do not have detectable α8 mRNA (23) or α8 protein (21). The only other AChR gene product known to bind αBgt is the muscle AChR α1 gene product. The α1 subunit is known also to bind mAb 35 (21, 22), making it a strong candidate to be part of the αT/35-AChR if α1 expression were not confined to muscle. In primates an α1 variant appears to be expressed in a number of tissues (28). Accordingly, RNase protection assays were carried out with ciliary ganglion RNA to determine whether the α1 gene was expressed in the tissue. Surprisingly, significant amounts of α1 transcript were detected in RNA from

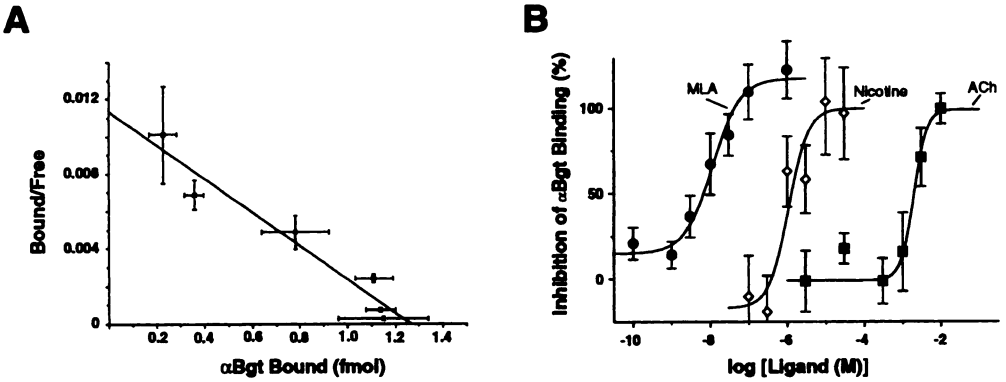

Fig. 2. Pharmacological profile of ciliary ganglion components that bind both αBgt and mAb 35. Extracts from E17–18 ciliary ganglia were incubated in solid-phase immunoprecipitation assays using mAb 35 to tether αBgt-binding components. The equilibrium binding of ^{125}I-αBgt conducted over a 24-hr period at 4° was determined in the presence and absence of competing ligands. A, Scatchard analysis of ^{125}I-αBgt binding. Similar results were obtained in two additional experiments, yielding values of 1.0 ± 0.1 nM (mean ± standard error) for the K_d and 1.1 ± 0.1 fmol/ciliary ganglion for the B_{max}. B, Inhibition by the indicated concentrations of methyllycaconitine (●), nicotine (◇), and ACh (■) of ^{125}I-αBgt binding during a 24-hr period at 4°. Values are expressed as a percentage of the reduction in 10 nM ^{125}I-αBgt binding caused by 1 μM unlabeled αBgt and represent the mean ± standard error for three determinations for each ligand in the same experiment. Similar results were obtained in several additional experiments, and the results have been tabulated in Table 2.

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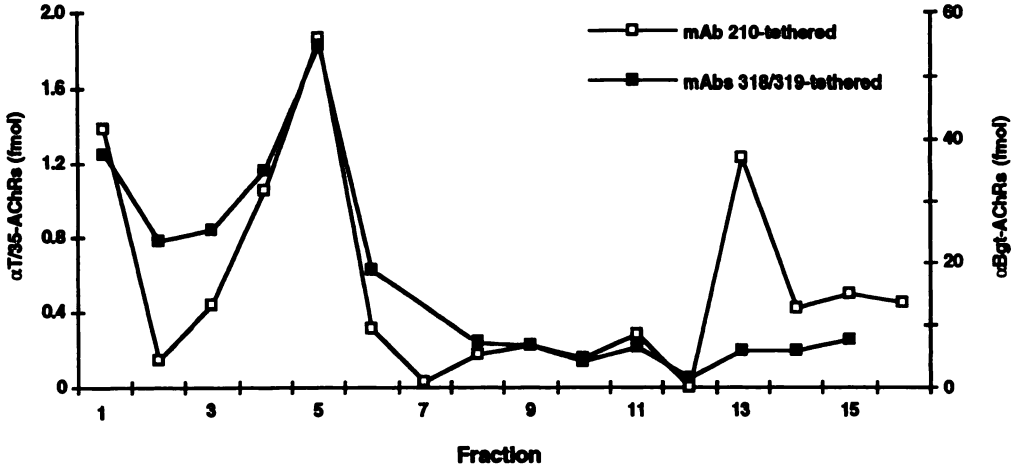

Fig. 3. Sucrose gradient analysis showing that the ciliary ganglion component recognized by both αBgt and mAb 35 (αT/35-AChRs) has the size expected of a fully assembled AChR. Extracts from E17–18 ciliary ganglia were fractionated by sucrose gradient sedimentation and the fractions were analyzed, by solid-phase immunoprecipitations, for the number of ^{125}I-αBgt binding sites retained by mAb 210 (αT/35-AChRs) (□; *left scale*) or mAbs 318/319 (αBgt-AChRs) (■; *right scale*). Sedimentation was from *right* to *left*; values represent specific binding normalized for the fraction volume assayed and are all from the same gradient. Similar results were obtained in three experiments using either mAb 210 or mAb 35 to tether αT/35-AChRs.

both E11 and E15 ciliary ganglia (Fig. 4). Quantification of the amount present in one experiment with E15 RNA indicated an α1 transcript level equivalent to 1–5% of that found for α7 mRNA, the most abundant AChR gene transcript in the ganglia. The level was less than one third of those observed for the less abundant α5, β2, and β4 transcripts.

Immunoblot analysis of αT/35-AChRs for α1 protein. Because small amounts of α1 mRNA were detected in ciliary ganglia, it was essential to determine whether α1 protein was present in the αT/35-AChRs. To isolate material for the required immunoblot analysis, ciliary ganglion extracts were depleted simultaneously with mAb 318/319-Actigel (to remove α7-containing αBgt-AChRs) and mAb A3–1- and B4–1-Actigels (to remove mAb 35-AChRs). The immunodepletions did not remove αT/35-AChRs but did reduce the amount of nonspecific adsorption of receptor protein to affinity resins that otherwise occurred in subsequent steps. As a negative control, extracts were also depleted with mAb 35-Actigel (to remove αT/35-AChRs). In both cases the recovered extracts were then adsorbed with αBgt-Actigel, and the adsorbed proteins were eluted with SDS sample buffer and analyzed on immunoblots probed with mAb 61, which recognizes the α1 protein.

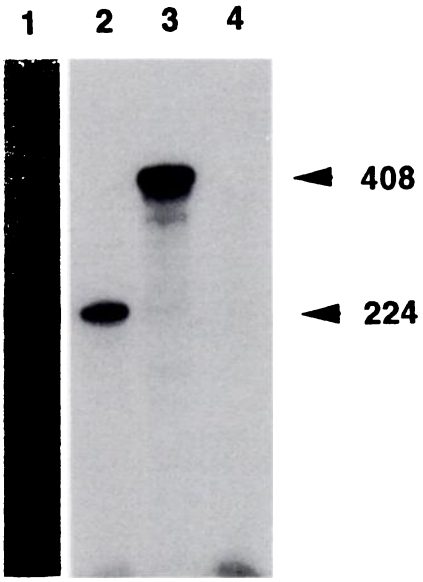

Fig. 4. RNase protection assays, detecting a small amount of α1 transcript in ciliary ganglion RNA. RNA from chick ciliary ganglia (E15, 25 μg) (*lane 1*), pectoral muscle (E15, 5 μg) (*lane 2*), and liver (E17–18, 25 μg) (*lane 4*) was subjected to RNase treatment in the presence of a ^{32}P-labeled antisense riboprobe (*lane 3*) specific for the α1 sequence. A 224-base pair band was protected in both the ciliary ganglia and muscle lanes, whereas none was protected by liver RNA. All samples were run on the same gel, and intervening lanes were removed to show the lanes of interest. Similar results were obtained in a second experiment using E11 ciliary ganglion RNA. *Arrowheads* mark the positions of the 408-base pair riboprobe and the 224-base pair protected band.

No detectable α1 protein was present in the αT/35-AChR sample, although the sample contained 30 ganglion equivalents of material (about 30 fmol) (Fig. 5A, *lane 1*). The aggregated material at the top of the running gel was nonspecifically retained in the immunoadsorption, because it was present in the negative control sample as well (Fig. 5A, *lane 2*). As positive controls to demonstrate the sensitivity of the mAb, samples of muscle AChRs were co-purified on αBgt-Actigel and analyzed in parallel on the blots (Fig. 5A, *lanes 3-5*). mAb 61 was able to detect α1 protein when as little as 1 fmol of affinity-purified muscle AChR was applied to the gel lane (Fig. 5A, *lane 3*). The overexposures necessary to reveal such small amounts of α1 protein also brought out nonspecific binding visible as higher molecular mass bands in several lanes. Similar results were obtained in three other experiments. A fourth revealed a tiny amount (<1 fmol) of α1 protein in the αT/35-AChR sample, but it was far less than the amount of receptor in the sample (about 20 fmol). A muscle α1 signal of the appropriate size was still apparent when 1 fmol of the muscle receptor was added to the αT/35-AChR sample and examined on blots (data not shown), providing assurance that, had an α1 subunit been present as a normal constituent of αT/35-AChRs, it would have been detected.

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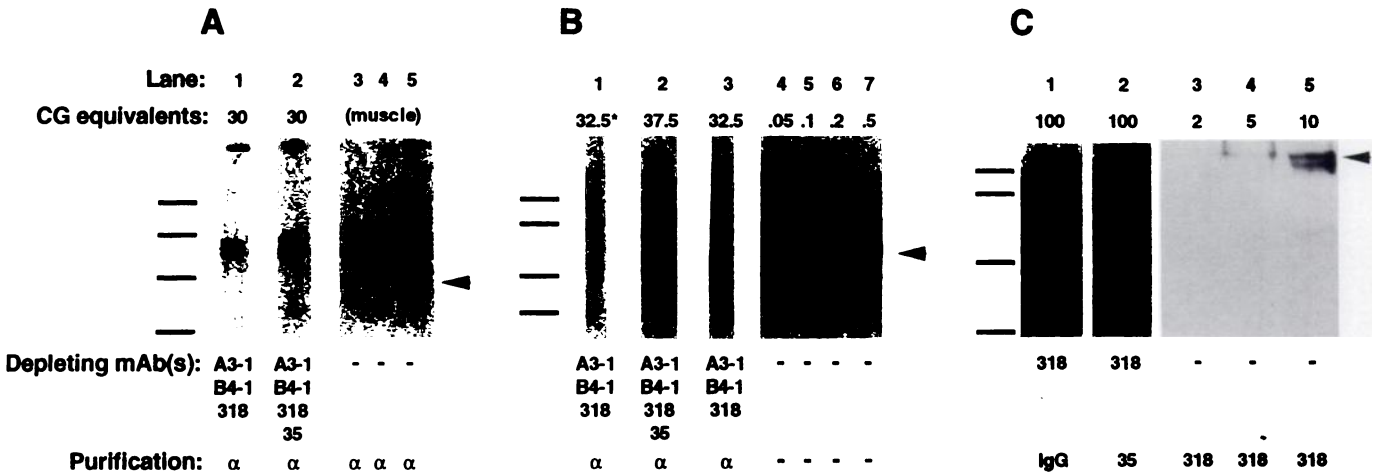

Fig. 5. Immunoblots demonstrating the lack of significant α1, α5, and α7 protein in αT/35-AChRs. A, Tests for the presence of α1 protein. Extracts were immunodepleted simultaneously with mAb 318-, A3–1-, and B4–1-Actigels to reduce background binding and were then incubated with αBgt-Actigel (α) to adsorb αT/35-AChRs. Samples were eluted in SDS sample buffer, immunoblotted, and probed with mAb 61 to detect α1 protein. No α1 protein was detected either in αT/35-AChRs (*lane 1*) or in the negative control immunodepleted with mAb 35-Actigel along with the other mAb-Actigels before adsorption with αBgt-Actigel (*lane 2*), although each contained 30 ganglion equivalents of material. The higher molecular mass material (50–60 kDa and the top of the running gel) in *lane 1* was nonspecific, because it was present in *lane 2* as well. mAb 61 detected α1 protein in as little as 1 fmol of E11 muscle AChRs adsorbed to and eluted from αBgt-Actigel (*arrowhead*; *lanes 3-5*, containing 1, 2, and 5 fmol, respectively, of muscle AChR). (The identity of the band at 95–100 kDa in the muscle samples is unknown; it was absent from ciliary ganglion samples.) Similar results were obtained in four additional experiments; only one sample of αT/35-AChRs displayed a small amount of α1 protein (about 1 fmol in 20 ganglion equivalents). Mixing 1 fmol of muscle AChR with 20 ganglion equivalents of αT/35-AChRS did not prevent detection of the muscle α1 protein on immunoblots (data not shown), suggesting that degradation did not obscure the component in ganglionic samples. B, Tests for α5 protein. αT/35-AChRs and negative controls were prepared as in A, immunoblotted, and probed with mAb 268 to detect α5 protein. A small amount of α5 protein (*arrowhead*) was present in the αT/35-AChR sample (*lane 3*) and appeared specific, because it was absent from the negative control (*lane 2*) and did not appear when primary mAb was omitted (*lane 1*). More than 30 ganglion equivalents of αT/35-AChRs contained less α5 protein than 0.1 ganglion equivalent of mAb 35-AChRs. Similar results were obtained in three additional experiments. Positive controls were produced by immunoblotting of extracts and probing with mAb 268 (0.05–0.5 ganglion equivalent, in *lanes 4-7*, as indicated). C, Tests for α7 protein. Extracts were immunodepleted with mAb 318-Actigel to remove αBgt-AChRs and were then adsorbed with mAb 35-Actigel to collect αT/35-AChRs. Bound protein was eluted at low pH, immunoblotted, and probed with mAb 318 (*lane 2*). As a negative control, IgG-Actigel was substituted for mAb 35-Actigel in the purification (*lane 1*). Positive controls were obtained by adsorbing extracts with mAb 318-Actigel, eluting the protein at low pH, immunoblotting, and probing with mAb 318 (2–10 ganglion equivalents, in *lanes 3-5*, as shown). Sample preparation (heating) produced aggregation of the α7 protein (*arrowhead*). Although specific signal could be detected in as few as 2 ciliary ganglion equivalents (at darker exposures), none was apparent in as many as 100 ganglion equivalents of αT/35-AChRs. Similar results were obtained in two additional experiments. In A and B, the tops of the lanes coincide with the start of the running gel; in C, part of the stacking gel is included to demonstrate that no aggregated material was overlooked. Intervening lanes containing molecular mass markers or irrelevant samples were removed in each panel. *Lines*, 97, 66, 45, and 31 kDa (*top* to *bottom*) (A and B) or 140, 87, 48, and 33 kDa (C). *CG equivalents*, the number of solubilized ganglia contained in the aliquot applied to the lane (except in A, *lanes 3-5*, which received muscle extract); *Depleting mAb(s)*, mAb-Actigels used (simultaneously when more than one was needed) to deplete extract samples; *Purification*, αBgt-Actigel (α), IgG-Actigel (*IgG*), or mAb-Actigel (*35* and *318*) used to collect samples before analysis.

Immunoblot analysis for α5 protein. The absence of α1 protein in αT/35-AChRs motivated a search for other subunit candidates. One remaining candidate was the α5 gene product. Of the five neuronal AChR genes known to be expressed in ciliary ganglia, the solid-phase immunoprecipitations with subunit-specific mAbs provided evidence that at least four were unlikely to contribute subunits to the receptor. These were the α3, β2, β4, and α7 genes, because mAbs specific for each of the encoded proteins were unable to bind and retain the αT/35-AChR. The α5 gene product could not be excluded in this manner, because the only mAbs that are capable of immunoprecipitating it (mAbs 35 and 210) also react with other gene products (21). Reasons for suspecting that the α5 protein might be present in αT/35-AChRs include the following: (a) the protein is known to be present in ciliary ganglion neurons, (b) it is capable of binding mAb 35, and (c) at least one report (29) indicates that it may have some affinity for αBgt.

When the αT/35-AChR was isolated as described above and analyzed on immunoblots with mAb 268, which is specific for the α5 subunit, very little α5 protein was detected (Fig. 5B, *lane 3*). The amount present in >30 ganglion equivalents of αT/35-AChRs (about 30 fmol) was equivalent to the amount of α5 protein in about 0.1 ganglion equivalent of mAb 35-AChRs (about 0.5 fmol) (Fig. 5B, *lanes 4–7*). The significance of a minor amount of α5 in the αT/35-AChR sample is unclear. Its presence appears specific, because it was absent from the negative control, but the amount is far too small to be consistent with a regular subunit of αT/35-AChRs, i.e., one or more copies/receptor. In one experiment the blot was also probed with mAbs 35 and 210, to determine whether a new protein could be identified. Both mAbs faintly detected a component only at the position of α5 (data not shown); the relative intensity of the signals was that expected for recognition of α5 on immunoblots with mAbs 35, 210, and 268 (21). Failure to detect more α5 protein in αT/35-AChRs is consistent with the observation that most, if not all, of the α5 protein sedimenting as part of a 10 S species on sucrose gradients is present as a subunit of mAb 35-AChRs containing α3 and β4 subunits (18).

Immunoblot analysis for α7 protein. The immunoprecipitation experiments illustrated in Fig. 1 provided direct

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evidence that αT/35-AChRs do not contain α7 subunits, and there is no precedence for α7 protein binding mAb 35, as do the putative receptors. Nonetheless, because none of the other known neuronal AChR gene products remained a candidate for a subunit of αT/35-AChRs, it seemed advisable to confirm the absence of α7 protein by a second method. Accordingly, immunoblot analysis of αT/35-AChRs was carried out using mAb 318 to detect α7 protein. In this case the experimental samples were prepared by first removing the α7-containing αBgt-AChRs with mAb 318-Actigel, to reduce nonspecific adsorption; the procedure did not deplete αT/35-AChRs. The αT/35-AChRs were then collected by adsorption to mAb 35-Actigel. Negative control samples were prepared by adsorbing extracts first with mAb 318-Actigel, as done for the experimental samples, and then readsorbing the depleted extract with IgG-Actigel, to assess nonspecifically retained residual α7 protein. Analysis of the experimental samples and negative controls revealed no detectable α7 protein specifically adsorbed, despite loading of 100 ganglion equivalents (about 100 fmol) of αT/35-AChRs onto the gel lane (Fig. 5C, *lane 2*). The sample preparation (heating) was designed to promote α7 aggregation as a means of increasing the sensitivity of antibody detection. Had α7 protein been present, it would have migrated as a large molecular mass species, as it did in the positive controls performed with α7-containing αBgt-AChRs eluted from the mAb 318-Actigel. The method was sensitive enough to reveal α7 protein in as few as 10 fmol of receptor. Even if the α7 protein had migrated as a monomer in the 50-kDa range (11), it should have been readily detectable with the methods used here were it a major subunit of αT/35-AChRs as it is in αBgt-AChRs (data not shown).

The immunoblots indicate that αT/35-AChRs do not contain significant amounts of α1, α5, or α7 protein. In the case of α7, the conclusion was reached independently in immunoprecipitation experiments. The only other AChR gene products known to be present in ganglia are α3, β2, and β4 subunits, and they too appear to be absent from αT/35-AChRs. Ganglia lack α2, α4, α8, and β3 transcripts (23), and the immunoprecipitation experiments confirmed a lack of α8 subunits in αT/35-AChRs. A chicken α6 gene, for which sequence data are not yet available (2), and a rat α9 gene expressed in certain ectodermally derived tissues of nonneural crest origin (12) are unlikely to encode subunits with the properties observed here for αT/35-AChRs (see Discussion). None of the muscle AChR gene products, other than α1, binds αBgt. The strong implication of these results is that other neuronal AChR genes remain to be identified in chick and that one or some combination of them produces receptors capable of binding both αBgt and mAb 35.

Tissue distribution of αT/35-AChRs. Extracts were prepared from a number of embryonic neural tissues to examine the distribution of αT/35-AChRs. The solid-phase assay revealed significant amounts of a component with similar properties both in sympathetic ganglia, where the receptors are about 2% as abundant as the α7-containing αBgt-AChRs, and in dorsal root ganglia, where they are about 15% as abundant (Table 3). In ciliary ganglia they are about 4% as abundant as α7-containing αBgt-AChRs. Expressed per unit protein, the values are comparable, with levels in ciliary ganglia being slightly higher than those in sympathetic ganglia and with levels in dorsal root ganglia being the lowest (Table 3). No

TABLE 3

Tissue distribution of αT/35-AChRs

Tissue extracts were analyzed, by solid-phase immunoprecipitation assays, for ^{125}I-αBgt-binding components that could be tethered by mAb 35 (αT/35-AChRs) or by mAbs 318/319 (αBgt-AChRs). The results represent the mean ± standard error of at least four determinations in at least two separate experiments.

Tissue extract[a]	^{125}I-αBgt-binding components tethered by mAb 35	
	fmol/mg of protein	*Equivalent % of αBgt-AChRs*
CG	69.1 ± 4.8	4.6 ± 0.5
SG	18.1 ± 6.6	2.0 ± 0.8
DRG	42.8 ± 21.5	15.3 ± 10.0
Spinal cord	0.2 ± 0.2	0.3 ± 0.3
Cerebellum	0.2 ± 0.2	1.2 ± 1.2
Brain	ND[b]	0.5 ± 0.5
Retina	ND[b]	0.3 ± 0.3

[a] CG, ciliary ganglion; SG, sympathetic ganglion; DRG, dorsal root ganglion.
[b] ND, protein concentrations not determined.

αT/35-AChRs were detected in extracts prepared from central nervous system tissues, including the spinal cord, brain, retina, and cerebellum.

Developmental regulation of αT/35-AChRs in ciliary ganglia. Possible developmental regulation of αT/35-AChRs was assessed by quantifying the number of such components in extracts prepared from ganglia at various stages. The solid-phase assay indicated that αT/35-AChRs are present at E8, increase slightly by E11, and reach maximum levels at E14–15 (Fig. 6). In this series of experiments the ratio of αT/35-AChRs to α7-containing αBgt-AChRs remained at about 3% at all times examined, indicating that the developmental regulation was comparable in time for the two species.

Discussion

Although the putative AChR identified here is likely to be a minor species in the vertebrate nervous system, its significance is derived principally from three features. First, it represents a new species capable of binding αBgt, indicating

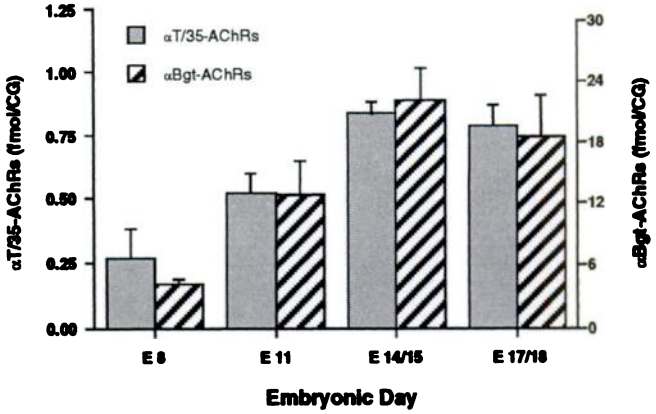

Fig. 6. Relative amounts of αT/35-AChRs and αBgt-AChRs in ciliary ganglia during development. Ciliary ganglion extracts prepared from chick embryos of the indicated ages were assayed by solid-phase immunoprecipitations for αT/35-AChRs and αBgt-AChRs, by tethering components with mAb 35 (▩) and mAbs 318/319 (▨), respectively, and quantifying them with ^{125}I-αBgt. The results are presented as fmol of αBgt binding/ganglion (*left scale*, αT/35-AChRs; *right scale*, αBgt-AChRs) and represent the mean ± standard error for three experiments (three trials in each experiment) in each case, except for E17/18, which involved two experiments. Although αT/35-AChRs are less abundant than αBgt-AChRs, they show similar changes in amounts over the time period examined.

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that such receptors are more diverse in composition than previously recognized. Second, its presence in ciliary ganglia indicates, in conjunction with previous work, that these neurons are likely to produce at least four distinct classes of AChRs. Third, the putative AChR identified here does not contain detectable levels of subunits encoded by known AChR genes, including those present in other AChRs recognized by αBgt. The implication is that additional AChR genes remain to be cloned.

Previous studies demonstrated that ciliary ganglion neurons express αBgt-AChRs, an abundant class of receptors that contain the α7 gene product (11, 27). It was recognized at the time that the presence of a minor species binding both αBgt and mAb 35 would have gone undetected with the assays available if the minor species was present in <5% the abundance of the αBgt-AChRs. The present results, using a sensitive solid-phase immunoprecipitation assay, identified the αT/35-AChRs even though they are 3–4% as abundant as αBgt-AChRs in the ganglia.

The newly identified putative receptor, the αT/35-AChR, is likely to be a *bona fide* AChR even though a receptor function has yet to be identified for it. The strongest evidence is provided by the nicotinic pharmacology of the component. Additional evidence is provided by its size, as inferred from sucrose gradient sedimentation, and by its ability to bind both mAb 35 and αBgt, which, to date, have been shown to bind only to AChR proteins.

The absence of known gene products in the αT/35-AChRs is intriguing. Immunoprecipitations with subunit-specific antibodies have been widely used to examine the subunit composition of proteins, and immunoblots of the purified components have provided verification of subunit identity. This strategy has been used successfully to determine the subunit composition of ciliary ganglion AChRs containing each of the five neuronal AChR gene products known to be expressed in ganglia (11, 18). The same antibodies have been unsuccessful in identifying subunits of αT/35-AChRs. mAbs specific for the α3, α7, β2, and β4 subunits immunoprecipitate ciliary ganglion AChRs containing those gene products but do not immunoprecipitate αT/35-AChRs. Of the remaining neuronal AChR genes that have been cloned, only α5 is expressed in ganglia (23). Although mAb 35 recognizes the α5 gene product, among others, and does immunoprecipitate αT/35-AChRs, immunoblot analysis reveals little α5 gene product in the putative receptors. This finding is consistent with studies showing that essentially all of the α5 gene product sedimenting in sucrose gradients at the position occupied by fully assembled AChRs is associated with α3 and β4 subunits (18), as expected for mAb 35-AChRs but not for αT/35-AChRs.

Because αT/35-AChRs have a pharmacology similar to that of α7-containing αBgt-AChRs, it seemed important to corroborate the immunoprecipitation studies showing that the former lacked α7 protein. This was achieved by immunoblot analysis. Immunoprecipitations also confirmed the absence of α8 protein from the receptors, consistent with the lack of α8 mRNA in the ganglia (23). Two other genes deserving comment are the chicken α6 and rat α9 genes. No sequence information is available for the chicken α6 gene (2), preventing it from being tested here. If the chicken gene is similar in sequence, however, to the rat homologue (30, 31), it is very unlikely to be responsible for either the αBgt or mAb 35 binding of αT/35-AChRs, because the rat gene conspicuously lacks the critical amino acid residues needed for such binding (32, 33). Similar considerations apply to the α9 gene. If the chicken homologue exists, it is unlikely to account for the mAb 35 binding of αT/35-AChRs, because the rat α9 gene lacks essential amino acids for mAb 35 binding and because the pharmacology of rat α9 AChRs expressed in *Xenopus* oocytes differs markedly, in the ligand binding profile, from that observed here for αT/35-AChRs. Most notable are the 2–3-order of magnitude differences in K_i values for nicotine and strychnine observed for the two receptor species and the fact that the αBgt blockade of α9 receptors is readily reversible (12) (Table 2). The α9 gene is also an unlikely candidate because it is expressed principally in structures derived from ectoderm of non-neural crest origin (12), whereas αT/35-AChRs are present in sympathetic, parasympathetic, and sensory ganglia.

One gene product that should bind both mAb 35 and αBgt is the α1 gene product. Recent studies showing that some neuronal AChR genes can be expressed in developing muscle tissue (19) suggest that the tissue-specific division of AChR gene expression may be less categorical than previously thought. The present results with RNase protection experiments demonstrate that the α1 gene is expressed in the ciliary ganglia, although at low levels. Despite this surprising finding, immunoblot analysis failed to detect α1 protein in αT/35-AChRs. Because α1 protein would have co-purified with αT/35-AChRs, the inability to visualize it on the immunoblots indicates that very little is present. The source of α1 mRNA in the ganglia and the possible presence of α1 protein have yet to be established.

The cellular distribution of αT/35-AChRs is also an interesting issue. In principle, the putative receptors could be present on ganglionic neurons, non-neuronal cells in the ganglia, or preganglionic nerve terminals. If present on terminals, the receptors might serve an auto-feedback function influencing ACh release. If present on non-neuronal cells, the receptors would add to a growing list of ligand-gated ion channels of unknown physiological significance found on such cells (34, 35). Preganglionic terminals are unlikely to represent the primary source of the putative receptors in the ganglia, because ciliary ganglion neurons in culture contain about the same number of such components as found *in vivo*.[1] Non-neuronal cells are also unlikely to be the major supplier of αT/35-AChRs, because the putative receptors constitute about 20% of the total mAb 35 binding in the ganglia but neither immunofluorescence nor electron microscopy with antibody probes has detected mAb 35 binding associated with non-neuronal cells in ganglia (36). It seems probable that αT/35-AChRs are present on ganglionic neurons. The receptors would then constitute a fourth class of AChRs, distinguishable by subunit composition, produced by the cells *in vivo* (18).

A clear implication of the present results is that αT/35-AChRs contain subunits encoded by one or more genes yet to be discovered. The encoded gene products could contribute to other AChR species as well, although presumably in combinations different from the one found in αT/35-AChRs. The alternative possibility is that αT/35-AChRs are made up of subunits encoded by known AChR genes expressed in the ciliary ganglia but the subunits are modified in ways that

[1] P. C. Pugh and D. K. Berg, unpublished observations.

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prevent their detection by the methods used. This latter possibility seems less likely, because confirmation was sought with immunoblot analysis and positive controls were included to demonstrate the competence of the antibody being used. Nonetheless, the question cannot be definitively resolved until the actual subunits of αT/35-AChRs are identified. Novel subunit modifications would themselves raise questions regarding their physiological relevance. The larger question deserving attention, however, is what role αT/35-AChRs play in the nervous system.

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References

1. Clarke, P. B. S. The fall and rise of neuronal α-bungarotoxin-binding proteins. *Trends Pharmacol. Sci.* **13**:407–413 (1992).
2. Sargent, P. B. The diversity of neuronal nicotinic acetylcholine receptors. *Annu. Rev. Neurosci.* **16**:403–443 (1993).
3. Zhang, Z.-w., S. Vijayaraghavan, and D. K. Berg. Neuronal acetylcholine receptors that bind α-bungarotoxin with high affinity function as ligand-gated ion channels. *Neuron* **12**:167–177 (1994).
4. Vijayaraghavan, S., P. C. Pugh, Z.-w. Zhang, M. M. Rathouz, and D. K. Berg. Nicotinic receptors that bind α-bungarotoxin on neurons raise intracellular free Ca^{2+}. *Neuron* **8**:353–362 (1992).
5. Pugh, P. C., and D. K. Berg. Neuronal acetylcholine receptors that bind α-bungarotoxin mediate neurite retraction in a calcium-dependent manner. *J. Neurosci.* **14**:889–896 (1994).
6. Jacob, M. H., and D. K. Berg. The ultrastructural localization of α-bungarotoxin binding sites in relation to synapses on chick ciliary ganglion neurons. *J. Neurosci.* **3**:260–271 (1983).
7. Loring, R. H., L. M. Dahm, and R. E. Zigmond. Localization of α-bungarotoxin binding sites in the ciliary ganglion of the embryonic chick: an autoradiographic study at the light and electron microscopic level. *Neuroscience* **25**:645–660 (1985).
8. Couturier, S., D. Bertrand, J.-M. Matter, M.-C. Hernandez, S. Bertrand, N. Millar, S. Valera, T. Barkas, and M. Ballivet. A neuronal nicotinic acetylcholine receptor subunit (α7) is developmentally regulated and forms a homo-oligomeric channel blocked by α-BTX. *Neuron* **5**:847–856 (1990).
9. Schoepfer, R., W. G. Conroy, P. Whiting, M. Gore, and J. Lindstrom. Brain α-bungarotoxin binding protein cDNAs and MAbs reveal subtypes of this branch of the ligand-gated ion channel gene superfamily. *Neuron* **5**:35–48 (1990).
10. Keyser, K. T., L. R. Britto, R. Schoepfer, P. Whiting, J. Cooper, W. Conroy, A. Brozozowska-Prechtl, H. J. Karten, and J. Lindstrom. Three subtypes of α-bungarotoxin-sensitive nicotinic acetylcholine receptors are expressed in chick retina. *J. Neurosci.* **13**:442–454 (1993).
11. Vernallis, A. B., W. G. Conroy, and D. K. Berg. Neurons assemble acetylcholine receptors with as many as three kinds of subunits and can segregate subunits among receptor subtypes. *Neuron* **10**:451–464 (1993).
12. Elgoyhen, A. B., D. S. Johnson, J. Boulter, D. E. Vetter, and S. Heinemann. α9: an acetylcholine receptor with novel pharmacological properties expressed in rat cochlear hair cells. *Cell* **79**:705–715 (1994).
13. Anand, R., X. Peng, J. Ballesta, and J. Lindstrom. Pharmacological characterization of α-bungarotoxin-sensitive acetylcholine receptors immunoisolated from chick retina: contrasting properties of α7 and α8 subunit-containing subtypes. *Mol. Pharmacol.* **44**:1046–1050 (1993).
14. Anand, R., X. Peng, and J. Lindstrom. Homomeric and native α7 acetylcholine receptors exhibit remarkably similar but non-identical pharmacological properties, suggesting that the native receptor is a heteromeric protein complex. *FEBS Lett.* **327**:241–246 (1993).
15. Conti-Tronconi, B. M., S. M. J. Dunn, E. A. Barnard, J. O. Dolly, F. A. Lai, and M. A. Raftery. Brain and muscle nicotinic acetylcholine receptors are different but homologous proteins. *Proc. Natl. Acad. Sci. USA* **82**:5208–5212 (1985).
16. Gotti, C., A. E. Ogando, W. Hanke, R. Schlue, M. Moretti, and F. Clementi. Purification and characterization of an α-bungarotoxin receptor that forms a functional nicotinic channel. *Proc. Natl. Acad. Sci. USA* **88**:3258–3262 (1991).
17. Gotti, C., W. Hanke, W.-R. Schlue, L. Briscini, M. Moretti, and F. Clementi. A functional α-bungarotoxin receptor is present in chick cerebellum: purification and characterization. *Neuroscience* **50**:117–127 (1992).
18. Conroy, W. G., and D. K. Berg. Neurons can maintain multiple classes of nicotinic acetylcholine receptors distinguished by different subunit compositions. *J. Biol. Chem.* **270**: 4424–4431 (1995).
19. Corriveau, R. A., S. J. Romano, W. G. Conroy, L. Oliva, and D. K. Berg. Expression of neuronal acetylcholine receptor genes in vertebrate skeletal muscle during development. *J. Neurosci.* **15**: 1372–1383 (1995).
20. Cheng, Y.-C., and W. H. Prusoff. Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. *Biochem. Pharmacol.* **22**:3099–3108 (1973).
21. Conroy, W. G., A. B. Vernallis, and D. K. Berg. The α5 gene product assembles with multiple acetylcholine receptor subunits to form distinctive receptor subtypes in brain. *Neuron* **9**:679–691 (1992).
22. Tzartos, S., D. Rand, B. Einarson, and J. M. Lindstrom. Mapping of surface structure of *Electrophorus* acetylcholine receptor using monoclonal antibodies. *J. Biol. Chem.* **256**:8635–8645 (1981).
23. Corriveau, R. C., and D. K. Berg. Coexpression of multiple acetylcholine receptor genes in neurons: quantification of transcripts during development. *J. Neurosci.* **13**:2662–2671 (1993).
24. Chomczynski, P., and N. Sacchi. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**:156–159 (1987).
25. Ravdin, P. M., and D. K. Berg. Inhibition of neuronal acetylcholine sensitivity by α-toxins from *Bungarus multicinctus* venom. *Proc. Natl. Acad. Sci. USA* **76**:2072–2076 (1979).
26. Lindstrom, J., B. Einarson, and S. Tzartos. Production and assay of antibodies to acetylcholine receptors. *Methods Enzymol.* **74**:452–460 (1981).
27. Smith, M. A., J. Stollberg, J. M. Lindstrom, and D. K. Berg. Characterization of a component in chick ciliary ganglia that cross-reacts with monoclonal antibodies to muscle and electric organ acetylcholine receptor. *J. Neurosci.* **5**:2726–2731 (1985).
28. Talib, S., T. B. Okarma, and J. S. Lebkowski. Differential expression of human nicotinic acetylcholine receptor α subunit variants in muscle and non-muscle tissues. *Nucleic Acids Res.* **21**:233–237 (1994).
29. McLane, K. E., X. Wu, and B. M. Conti-Tronconi. Identification of a brain acetylcholine receptor α subunit able to bind α-bungarotoxin. *J. Biol. Chem.* **265**:9816–9824 (1990).
30. Boulter, J. Nucleotide sequence of a cDNA clone encoding the rat neuronal nicotinic acetylcholine receptor subunit gene *alpha6*. GenBank database entry, locus/accession number RATNARA6S/L08227 (1993).
31. Benson, D. A., M. Boguski, D. J. Lipman, and J. Ostell. GenBank. *Nucleic Acids Res.* **22**:3441–3444 (1994).
32. Saedi, M. S., R. Anand, W. G. Conroy, and J. Lindstrom. Determination of amino acids critical to the main immunogenic rection of intact acetylcholine receptors by *in vitro* mutagensis. *FEBS Lett.* **267**:55–59 (1990).
33. Chaturvedi, V., D. L. Donnelly-Roberts, and T. L. Lentz. Substitution of *Torpedo* acetylcoine receptor α1-subunit residues with snake α1- and rat nerve α3-subunit residues in recombinant fusion proteins: effect on α-bungarotoxin binding. *Biochemistry* **31**:1370–1375 (1992).
34. Jabs, R., F. Kirchloff, H. Kettenmann, and C. Steinhauser. Kainate activates $Ca^{(2+)}$-permeable glutamate receptors and blocks voltage-gated K^+ currents in glial cells of mouse hippocampal slices. *Pfluegers Arch.* **426**: 310–319 (1994).
35. Müller, T., J. M. Fritschy, J. Grosche, G. D. Pratt, H. Mohler, and H. Kettenmann. Developmental regulation of voltage-gated K^+ channel and $GABA_A$ receptor expression in Bergmann glial cells. *J. Neurosci.* **14**:2503–2514 (1994).
36. Jacob, M. H., J. M. Lindstrom, and D. K. Berg. Surface and intracellular distribution of a putative neuronal nicotinic acetylcholine receptor. *J. Cell Biol.* **103**:205–214 (1986).

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