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Human α7 Acetylcholine Receptor: Cloning of the α7 Subunit from the SH-SY5Y Cell Line and Determination of Pharmacological Properties of Native Receptors and Functional α7 Homomers Expressed in *Xenopus* Oocytes

XIAO PENG, MICHAEL KATZ, VOLODYMYR GERZANICH, RENÉ ANAND, and JON LINDSTROM

Department of Neuroscience, University of Pennsylvania Medical School, Philadelphia, Pennsylvania 19104–6074

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SUMMARY

The α-bungarotoxin-binding acetylcholine receptors from the human neuroblastoma cell line SH-SY5Y were found to cross-react with some monoclonal antibodies to α7 subunits of nicotinic acetylcholine receptors from chicken brain. The human α7 subunit cDNA from SH-SY5Y was cloned, revealing 94% amino acid sequence identity to rat α7 subunits and 92% identity to chicken α7 subunits. Native human α7 receptors showed affinities for some ligands similar to those previously observed with native chicken α7 receptors, but for other ligands there were large species-specific differences in binding affinity. These results paralleled properties of α7 homomers expressed in *Xenopus* oocytes. Human α7 homomers exhibited rapidly desensitizing, inwardly rectifying, agonist-induced, cation currents that triggered Ca^{2+}-sensitive Cl^- channels in the oocytes. A change in efficacy from partial agonist for chicken α7 homomers to full agonist for human α7 homomers was exhibited by 1,1-dimethyl-4-phenylpiperazinium. This result reveals a large species-specific pharmacological difference, despite small differences in α7 sequences. This is important for understanding the effects of these drugs in humans and for identifying amino acids that may contribute to the acetylcholine binding site, for analysis by *in vitro* mutagenesis. These results also characterize properties of native α7 receptors and α7 homomers that will provide criteria for functional properties expected of structural subunits, when these can be identified, cloned, and coexpressed with α7 subunits.

There are three branches of the nicotinic AChR gene family, 1) muscle AChRs that bind the competitive antagonist αBgt, 2) neuronal AChRs that do not bind αBgt, and 3) neuronal AChRs that do bind αBgt. Aspects of AChR structure have recently been reviewed (e.g., Refs. 1–4).

Recently two cDNAs, termed α7 and α8, have been identified as subunits of chicken neuronal AChRs that bind αBgt (5, 6), and the rat homologue of α7 has also been cloned (7). Although α7 AChRs have been purified from chicken brain, their complete subunit composition is not yet known (8, 9). Three subtypes of neuronal AChRs that bind αBgt have been identified, all of which may contain uncharacterized structural subunits, 1) α7 AChRs, which contain α7 subunits, 2) α8 AChRs, which contain α8 subunits, and 3) α7α8 AChRs, which contain both α7 and α8 subunits (10). In the chick, but not yet in mammals, α7 and α8 subunits have been immunohistologically localized and α7 has been found to predominate in brain, whereas α8 predominates in retina (10–13). Some localization has also been done by *in situ* hybridization (7, 14). Unlike other AChR subunits, α7 efficiently assembles into homomeric ACh-gated cation channels when expressed in *Xenopus* oocytes (6). This has proven to be a great experimental advantage for expression and *in vitro* mutagenesis experiments, which have provided information relevant to basic structural features of all receptors in this gene superfamily (15–19). We have previously examined the ligand binding and functional pharmacological properties of chicken α7 and α8 homomers and α7 and α8 native AChRs (20–23).

The human neuroblastoma SH-SY5Y is of sympathetic adrenergic ganglion origin (23) and, like chicken ganglionic neurons (24), expresses two types of nicotinic AChRs, 1) ganglionic AChRs, which are normally postsynaptic and are composed of α3, α5, β2, and β4 subunits, and 2) neuronal αBgt-binding AChRs, which are probably normally extrasynaptic (25).[1] The

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[1] X. Peng and J. Lindstrom, unpublished observations.

ABBREVIATIONS: AChR, acetylcholine receptor; αBgt, α-bungarotoxin; ACh, acetylcholine; DMPP, 1,1-dimethyl-4-phenylpiperazinium; mAb, monoclonal antibody; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; kb, kilobase(s).

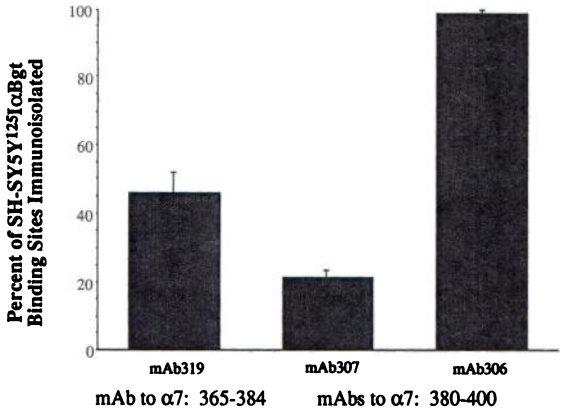

Fig. 1. Cross-reaction of mAbs to chicken α7 subunits with α7 AChRs in SH-SY5Y cells. Aliquots of detergent-solubilized cell extracts were assayed for binding of ^{125}I-αBgt after isolation of the binding component on DEAE in a small ion exchange column (this value was defined as 100%) or after isolation on mAb-coated microwells, as described in Materials and Methods. Each *bar* represents the mean ± standard deviation of triplicate determinations at 5 nM ^{125}I-αBgt. Other mAbs to chick α7 and α8 subunits (mAbs 305, 307, 309, 312, 318, and 320) bound <2.5% of the applied α7 AChRs.

α3-containing postsynaptic-type AChRs present in these cells have been shown to mediate agonist-sensitive cation flux, but this activity has not yet been detected for the neuronal αBgt-binding AChRs (25). This is similar to results obtained with chick ganglionic neurons, where the α3 AChR dominates the agonist-induced cation flux but agonist-induced Ca^{+2} influx can be detected through α7 AChRs under certain assay conditions (26). α7 homomers from chicks and rats and αBgt-blockable AChRs in rat hippocampal neurons are characterized by high Ca^{2+} conductance and rapid desensitization (7, 22, 27–29).

Here we report detection of α7 AChRs in the cell line SH-SY5Y, cloning of its α7 subunit cDNA, and comparison of the properties of α7 homomers expressed from this cDNA in *Xenopus* oocytes with properties of native human α7 AChRs from SH-SY5Y cells and previously published values for chicken α7 AChRs and α7 homomers.

Materials and Methods

Cell cultures. SH-SY5Y cells were initially provided by June Biedler and Barbara Spengler of the Sloan Kettering Institute for Cancer Research (23). Cultures were grown in a 1:1 mixture of Ham's F12 medium and Eagle's minimal essential medium containing 1% nonessential amino acids, supplemented with 10% fetal calf serum, in a 95% air/5% CO_2 humidified incubator at 37°. The cell monolayers were washed with phosphate-buffered saline, scraped, pelleted in a microfuge at 4°, and stored at −80° until used.

mAbs. mAb 306 and mAb 307 were prepared by using as antigen a mixture of affinity-purified native and denatured αBgt-binding AChRs from the brains of chickens and rats (5). Their epitopes were mapped, using synthetic peptides, to within the sequence 380–400 of chick α7 subunits (30). mAb 319 was prepared by using as antigen a bacterially expressed peptide fragment of α7 corresponding to its putative large cytoplasmic domain (5), and then its epitope was mapped more precisely, using synthetic peptides, to within residues 365–384 of chicken α7 subunits (30).

Binding assays. α7 AChRs from SH-SY5Y cells and α7 homomers expressed in *Xenopus* oocytes were solubilized in ~6 volumes of a solution containing 2% Triton X-100, 50 mM NaCl, 50 mM sodium phosphate buffer, pH 7.5, 5 mM EDTA, 5 mM EGTA, 2 mM phenylmethylsulfonyl fluoride, 5 mM benzamidine, and 5 mM iodoacetamide, by brief vortexing followed by 20 min of gentle rotation at 4° and then a 20-min centrifugation in a microfuge at 4°.

Binding to mAb 306-coated Immunolon 4 microwells (Dynatech) followed by overnight labeling with ^{125}I-αBgt, with or without competing ligands, was conducted as previously described for chicken α7 using mAb 319 or mAb 318 (20–22), using wells coated with 40 μg/ml purified IgG. Nonspecific binding was determined using wells not coated with mAbs.

Measurement of ^{125}I-αBgt binding to α7 AChRs immobilized on 150 μl of DE-52 ion exchange resin (Whatman) in compact reaction columns (United States Biochemicals) used 50 mM sodium phosphate buffer, pH 7.5, containing 0.5% Triton X-100. Aliquots (10 μl) of SH-SY5Y extract were labeled overnight at 4° with 5 nM ^{125}I-αBgt. After dilution in 100 μl of buffer the samples were applied to the column, and after 2 min the column was washed with 2 ml of buffer and then placed in a γ counter. Nonspecific binding was determined by incubation in the presence of 50 μM nicotine.

α7 cDNA cloning. Total RNA was isolated from SH-SY5Y cells as described by Chomczynski and Sacchi (31). Subsequently, poly(A)$^+$ RNA was isolated and used to construct a λZap II cDNA library (Stratagene). Approximately 3 × 10^6 plaques were screened with a rat α7 cDNA probe. Hybridizations were performed at 42° in 40% formamide, 5× Denhardt's solution (1× Denhardt's solution is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin), 0.5% sodium dodecyl sulfate, 5× SSPE (1× SSPE is 0.18 M NaCl, 0.01 M sodium phosphate buffer, pH 7.4, 1 mM EDTA), 0.15 mg/ml denatured salmon sperm DNA. Membranes were washed at 50° in 1× SSPE, 0.1% sodium dodecyl sulfate, and exposed to Kodak XAR-5 film. Nine separate positive clones were isolated and their inserts were rescued as described by Stratagene. Southern blot analysis demonstrated that the clones had an average insert size of 2.0 kb. One clone was selected and sequenced on both strands using the Sequenase version 2.0 kit (United States Biochemicals).

α7 expression in oocytes. Human α7 cDNA was subcloned into the *Bgl*II site of the expression vector pMXT (a derivative of SP64T) (32) using polymerase chain reaction methodology because it was found to be the easiest way to introduce vector-compatible restriction sites (*Bgl*II sites) at the 5′ and 3′ ends of the α7 cDNA. The reactions were carried out using *Pyrococcus furiosus* polymerase (Stratagene, San Diego) and the primers 5′-GGAGATCTTCAACATGCGCTGCTC-3′ and 5′-GGAGATCTGTGGCGTGTAATGCTG-3′ (*Bgl*II sites are underlined). The cDNA was amplified using *P. furiosus* polymerase in the buffer supplied by the manufacturer, using one cycle of 94° for 2 min, three cycles of 94° for 1 min, 50° for 2 min, and 72° for 3 min, 37 cycles of 92.5° for 1 min, 50° for 2 min, and 72° for 3 min, and one cycle of 72° for 5 min. The entire subcloned insert was sequenced and no errors were found. Subsequently, the human α7 cDNA was linearized with *Bam*HI and RNA was synthesized *in vitro* using SP6 RNA polymerase in conjunction with reagents from the Megascript kit (Ambion, Austin, TX). Oocytes were prepared for injections as described (33). Each oocyte was injected with 100 ng of the human α7 cRNA and then allowed to incubate at 18° in saline solution (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM HEPES, pH 7.6), containing 5% heat-inactivated horse serum (34), for 3 days before use.

Electrophysiology. Currents were measured using a standard two-microelectrode voltage-clamp amplifier (oocyte clamp OC-725; Warner Instrument Corp.) as previously described for chicken α7 and α8 homomers (29). Electrodes were filled with 3 M KCl and had resistances of 0.5–1.0 MΩ for the current electrode. All records were digitized (MacLab/2e interface; AD Instruments) and stored on an Apple Macintosh IIcx computer and were analyzed using SCOPE (AD Instruments) and AXOGRAPH software (Axon Instruments).

The recording chamber was continually perfused at a flow rate of 10 ml/min with saline solution (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.6). Application of drugs was performed using a set of eight glass tubes (internal diameter, 2 mm) ending in the bath 3 mm from the oocyte and connected to 10-ml syringes, which

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                  M  R  C  S  P  G  G  V  W  L  G  L  A  A  S  L  L  H  V  S  L  Q  G  E  F  Q
AGGAAGCTTTACAAGGAGCTGGTCAAGAACTACAATCCCTTGGAGAGGCCCGTGGCCAATGACTCGCAACCACTCACCGTCTACTTCTCCCTG
 R  K  L  Y  K  E  L  V  K  N  Y  N  P  L  E  R  P  V  A  N  D  S  Q  P  L  T  V  Y  F  S  L
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 S  L  L  Q  I  M  D  V  D  E  K  N  Q  V  L  T  T  N  I  W  L  Q  M  S  W  T  D  H  Y  L  Q
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 E  R  F  D  A  T  F  H  T  N  V  L  V  N  S  S  G  H  C  Q  Y  L  P  P  G  I  F  K  S  S  C
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 Y  I  D  V  R  W  F  P  F  D  V  Q  H  C  K  L  K  F  G  S  W  S  Y  G  G  W  S  L  D  L  Q
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 M  Q  E  A  D  I  S  G  Y  I  P  N  G  E  W  D  L  V  G  I  P  G  K  R  S  E  R  F  Y  E  C
                                                  M1
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 C  K  E  P  Y  P  D  V  T  F  T  V  T  M  R  R  R  T  L  Y  Y  G  L  N  L  L  I  P  C  V  L
                                                                  M2
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                                                                  M3
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 D  P  D  L  A  K  I  L  E  E  V  R  Y  I  A  N  R  F  R  C  Q  D  E  S  E  A  V  C  S  E  W
                                                  M4
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AACTTCGTGGAGGCCGTGTCCAAAGACTTTGCGTAACCACACTGGTTCTGTACATGTGGAAAACTCACAGATGGGCAAGGCCTTTGGCTTGGC
 N  F  V  E  A  V  S  K  D  F  A
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TCCTTTCCAATAATTGACTGGTGGAAGGAAAACAAAAAAAAAAAAA
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Fig. 2. Sequence of the human $\alpha 7$ subunit cDNA cloned from SH-SY5Y. This sequence has been given the EMBL accession number X70297.

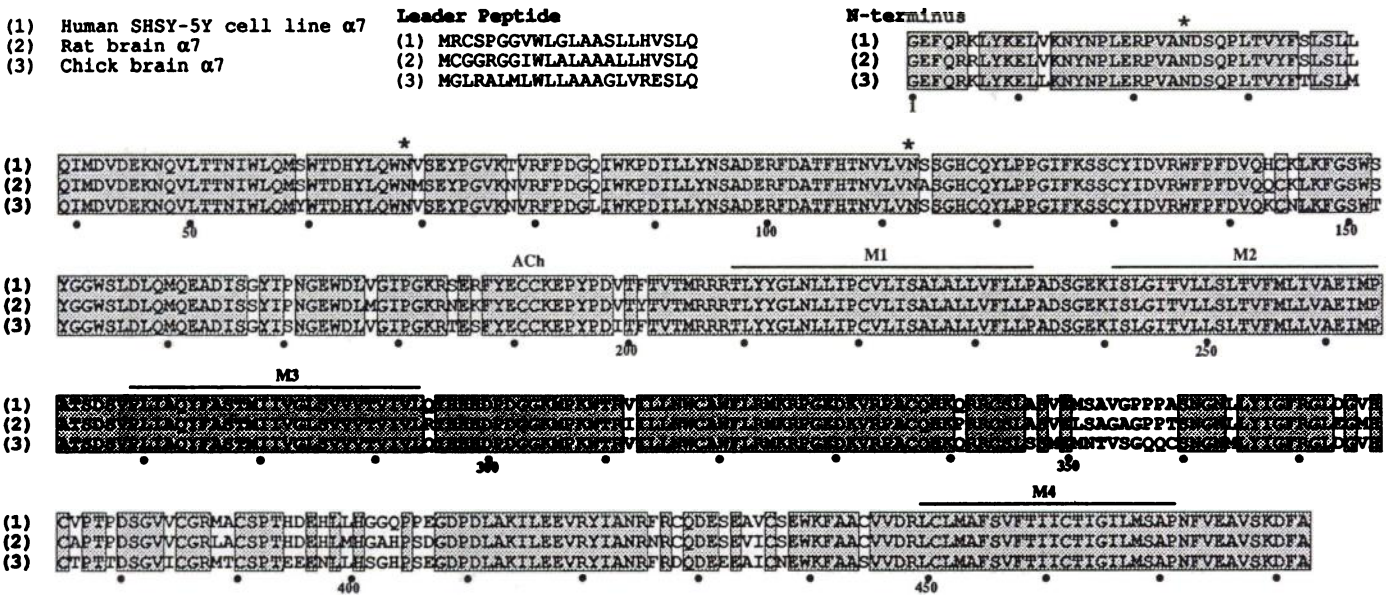

Fig. 3. Comparison of the amino acid sequence of the human $\alpha 7$ subunit with those of rat (7) and chick (5) subunits. *, Putative *N*-glycosylation sites; *ACh*, the adjacent, disulfide-linked, cysteine pair located near the ACh binding site of AChR α subunits (3); *M1-M4*, four putative transmembrane sequences typical of all AChR subunits.

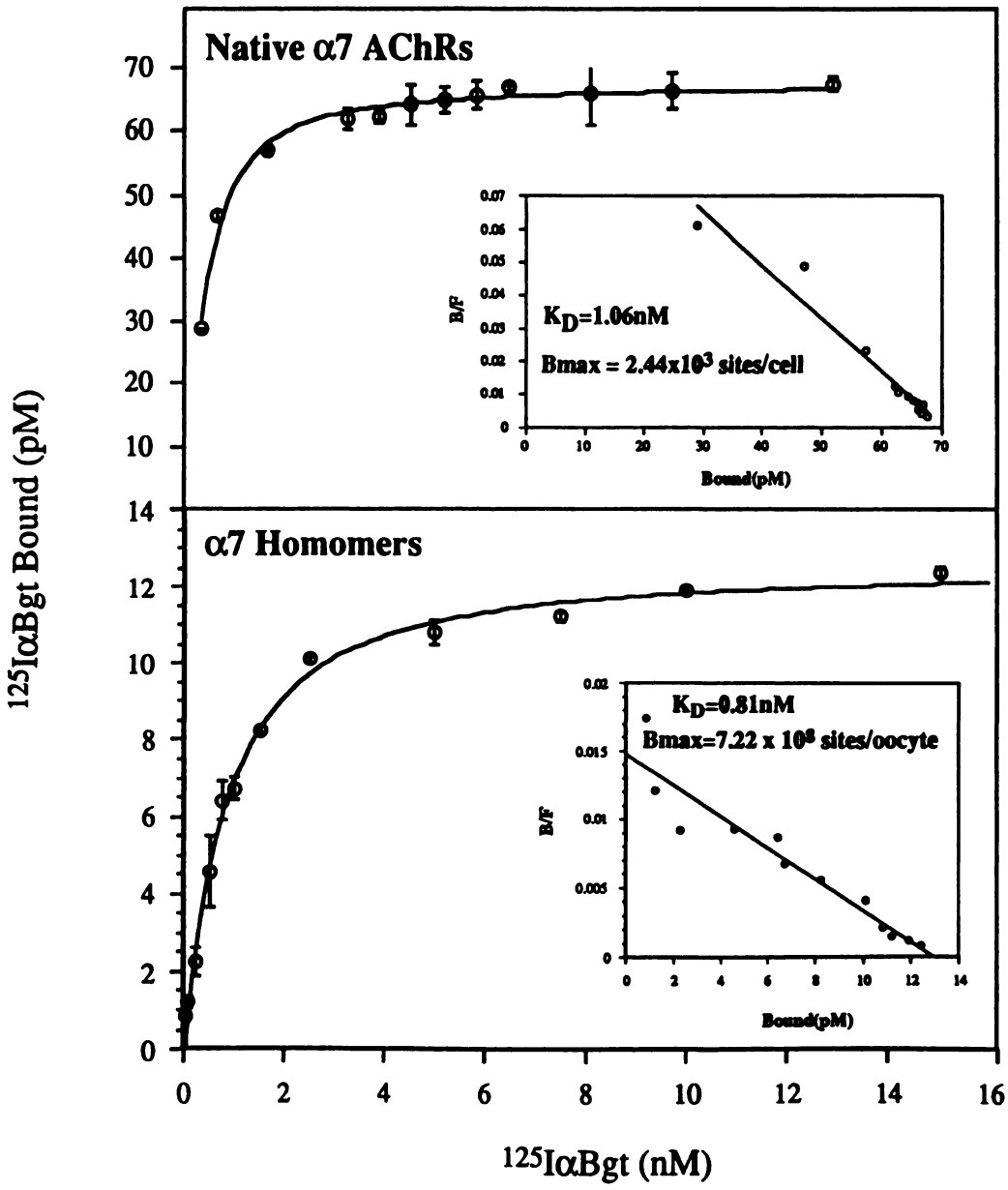

Fig. 4. Binding of ^{125}I-αBgt to native α7 AChRs from SH-SY5Y cells and to human α7 homomers expressed in *Xenopus* oocytes. Detergent-solubilized α7 was immobilized through its large putative cytoplasmic domain on microwells coated with mAb 306, and binding of ^{125}I-αBgt was determined after overnight incubation, as described in Materials and Methods. Each *data point* is the average of duplicates, and the *bars* indicate the range. Background values from wells not coated with mAb (usually <1% of the total) were subtracted from each point. *Insets*, Scatchard analysis of the binding data.

were placed 10 cm above the recording chamber and contained control and test solutions. Manual unclamping of the flexible tube connecting the syringe with the agonist solution to the perfusion glass tube directed at the oocyte resulted in appearance of current. The delay between the beginning of application (detected as a short inward deflection on some traces due to a mechanical artifact of unclamping of the connecting tube) and the appearance of current was typically 250–300 msec and apparently depended on the time needed for agonist to reach the oocyte. The application was terminated (typically after 2–5 sec) by clamping the connecting flow tube, and then the perfusion solution was effectively changed back to control due to the continuous flow through the recording chamber. Curare was applied by both bath application and addition to solutions containing agonist. Solutions used to test the effects of αBgt also contained bovine serum albumin (100 μg/ml) to minimize adsorption of αBgt to the plastic surfaces. αBgt was applied for 25 min in the bath before application of agonist.

Results and Discussion

Cross-reaction with mAbs specific for α7 subunits strongly suggested that SH-SY5Y cells express α7 AChRs. Eleven mAbs previously raised to α7 and α8 subunits from chickens (5) were tested by solid-phase radioimmunoassays for their ability to cross-react with the ^{125}I-αBgt-binding component in SH-SY5Y cells. Three mAbs to α7 subunits cross-reacted, as shown in Fig. 1. These mAbs are directed at two different epitopes on the putative large cytoplasmic domain of the α7 subunit (30), typically the most variable part of an AChR subunit between species (35). mAb 306 and mAb 307 were unique among the mAbs tested in having been raised against a mixture of affinity-purified brain αBgt-binding components from both chickens and rats, which thus contained both avian and mammalian

TABLE 1

Comparison of affinity for ligands of human and chicken native α7 AChRs and α7 homomers

The average n_H for all ligands and all AChRs was 0.91 ± 0.13 (range, 0.46–1.1).

	IC_{50}			
	Native human α7 AChRs	Native chicken α7 AChRs[a]	Human α7 homomers	Chicken α7 homomers[b]
		μM		
Agonists				
ACh	5.76 ± 2.2	160 ± 26	3.52 ± 2.1	55.5 ± 10
Carbamylcholine	17.3 ± 0.15	1580 ± 320	50.6 ± 1.1	557 ± 25
Cytisine	1.60 ± 0.15	2.02 ± 0.10	5.26 ± 2.2	0.17 ± 0.02
DMPP	4.36 ± 0.66	83.0 ± 6.0	20.5 ± 9.1	Not tested
L-Nicotine	2.60 ± 0.35	1.30 ± 0.60	2.50 ± 2.4	1.22 ± 0.04
Tetramethylammonium	28.8 ± 2.3	400 ± 110	6.27 ± 0.63	22.3 ± 4.2
Antagonists				
αBgt	0.00106 ± 0.000030	0.00191 ± 0.00020	0.000810 ± 0.000020	0.000162 ± 0.00008
Atropine	1360 ± 120	120 ± 12	1040 ± 290	330 ± 110
Curare	20.2 ± 6.2	7.30 ± 2.0	8.39 ± 0.9	11.1 ± 1.0
Strychnine	9.79 ± 4.6	9.90 ± 0.40	50.4 ± 3.4	15.3 ± 1.0

[a] Data from Ref. 21.
[b] Data from Ref. 20.

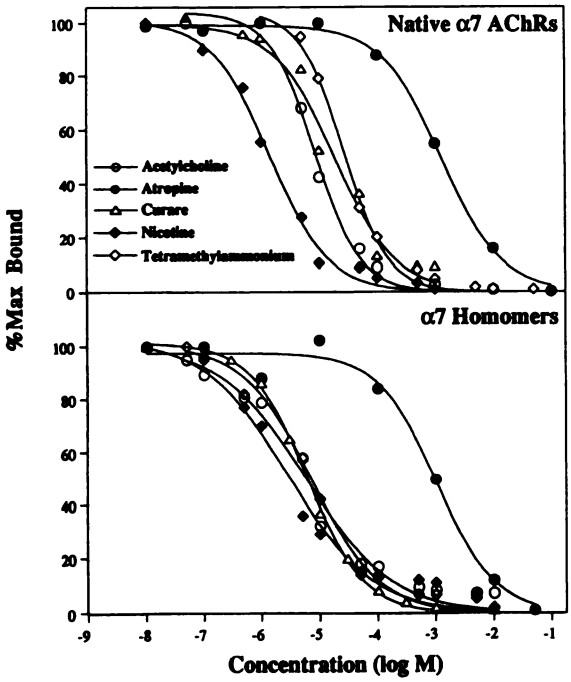

Fig. 5. Pharmacological properties of native α7 AChRs from SH-SY5Y cells and human α7 homomers expressed in *Xenopus* oocytes, measured by inhibition of ^{125}I-αBgt binding. Binding of 2 nM ^{125}I-αBgt to immunoisolated α7 was performed as in Fig. 4, with the addition of the indicated ligands during the overnight incubation. Representative *curves* from one experiment for some of the ligands tested are shown and each *point* is the average of duplicate determinations.

epitopes and may explain their cross-reaction with human α7 subunits. mAb 319 was raised to a bacterially expressed peptide corresponding to the putative large cytoplasmic domain of chicken α7. mAb 306 apparently had the highest affinity for the human α7 subunit, allowing it to retain all of the ^{125}I-αBgt binding sites present in an extract of SH-SY5Y cells, whereas reduced affinity apparently limited the extent of reaction with the other two mAbs. Thus, all subsequent ligand-binding studies used microwells coated with mAb 306 to immunoisolate human α7.

Proof that SH-SY5Y cells express α7 AChRs was provided

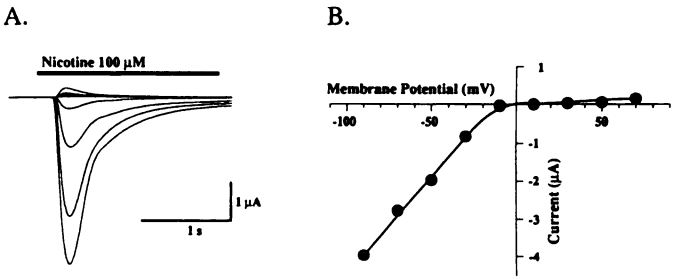

Fig. 6. Human α7 homomers expressed in *Xenopus* oocytes exhibit large, rapidly desensitizing responses and strong inward rectification. A, Responses to 2-sec applications of 100 μM nicotine are shown at membrane holding potentials from −90 to +70 mV, in 20-mV increments. B, The current/voltage relationship is plotted using the peak current amplitude at each holding potential.

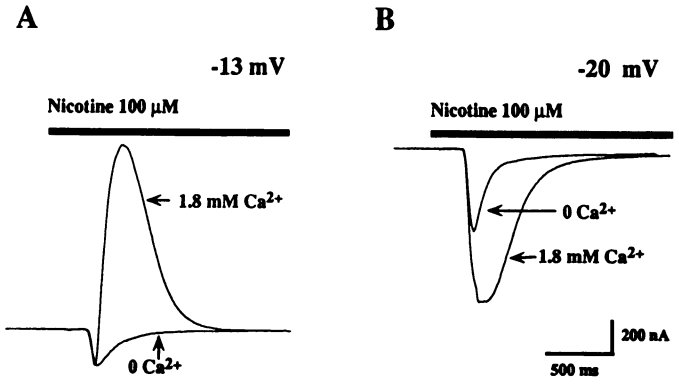

Fig. 7. Influx of Ca^{2+} through human α7 homomers triggers a large endogenous Ca^{2+}-dependent Cl^- current. A, At a holding potential of −13 mV, nicotine-induced currents in normal saline solution (1.8 mM Ca^{2+}) and 0 Ca^{2+} saline solution (saline solution with 0 mM Ca^{2+} plus 5 mM EGTA) are superimposed to emphasize the biphasic response in normal saline solution versus the small, rapidly desensitizing, inward current directly through α7 homomer channels measured in 0 Ca^{2+} saline solution. B, The same oocyte at a holding potential of −20 mV allows inward current flow in the same direction through both the Ca^{2+}-sensitive Cl^- channel and the α7 homomer channel. Cations enter through the α7 channel and Cl^- leaves the cell through the Cl^- channel.

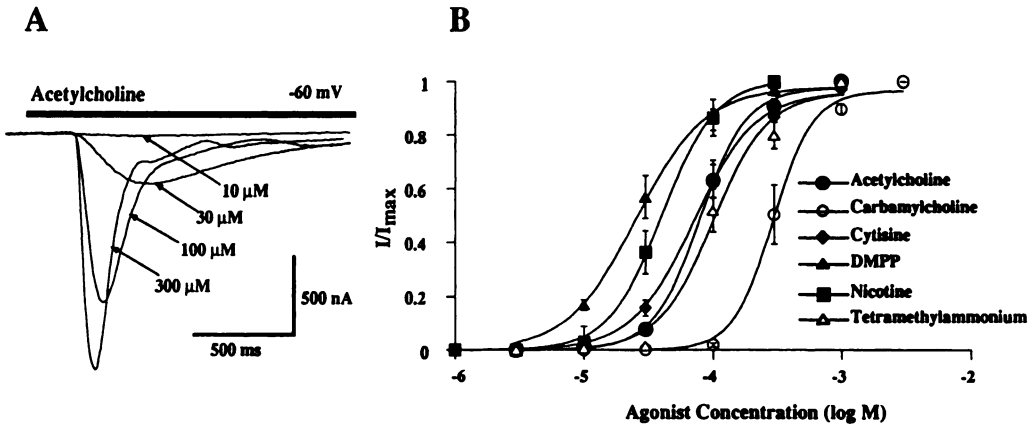

Fig. 8. Dose-response relationships for agonists with human α7 homomers. A, Superimposed responses of an oocyte to increasing concentrations of ACh show increasing maximum currents and increasing rates of desensitization. Because inactivation of the secondary Ca^{2+}-dependent Cl^- current (illustrated in Fig. 7) requires tens of seconds (42), the much faster rate of decay of the response observed must reflect primarily desensitization of α7 homomers. B, Dose-response curves for several agonists are shown normalized to the maximum response amplitude for each experiment. Each *point* is the average of three to five experiments at −60 mV. *Lines* are a fit of the Hill equation to the data.

TABLE 2

Comparison of functional pharmacological properties of human α7 homomers and chicken α7 homomers

	Human α7 homomers		Chicken α7 homomers[a]	
	EC_{50} or IC_{50}	n_H	EC_{50} or IC_{50}	n_H
	μM		μM	
Agonists				
ACh	79.2 ± 3.7	2.3 ± 0.2	110 ± 7	1.7
Carbamylcholine	296 ± 16	2.3 ± 0.6		
Cytisine	71.4 ± 3.9	1.8 ± 0.1	18 ± 2	1.4
DMPP	25.5 ± 1.4	1.6 ± 0.2	30 ± 3[b]	1.4
L-Nicotine	40.2 ± 1.7	2.0 ± 0.2	7.8 ± 3	1.7
Tetramethylammonium	101 ± 15	2.0 ± 0.6	800 ± 200	1.6
Antagonists				
αBgt	0.00220 ± 0.00020	1.3 ± 0.1	0.00030 ± 0.00002	1.3
Atropine	125 ± 2.5	0.9 ± 0.1	7.1 ± 0.5	0.8
Curare	0.70 ± 0.06	1.6 ± 0.2	0.14 ± 0.02	1.6
Strychnine	7.50 ± 0.50	1.0 ± 0.1	0.52 ± 0.03	1.3

[a] Data from Ref. 29.
[b] DMPP is a partial agonist for chicken α7 that at 1 mM produces a maximum of 3% of the full response to ACh.

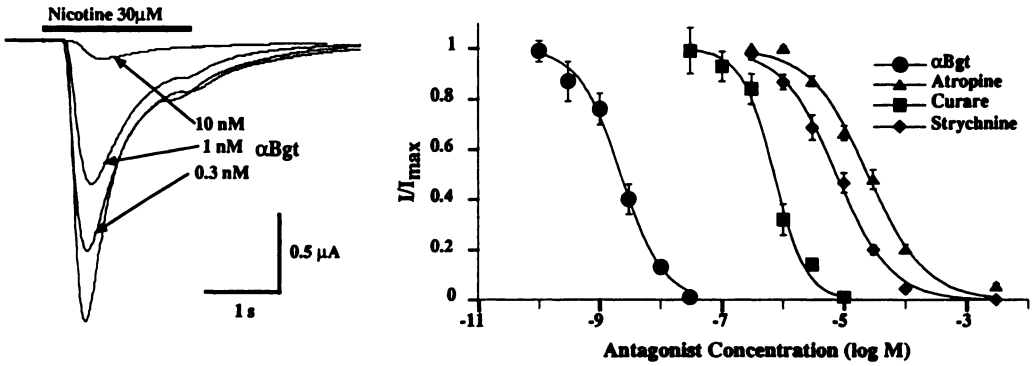

Fig. 9. Dose-response relationships for antagonists with human α7 homomers. *Left*, increasing concentrations of αBgt, applied for 25 min at each concentration, increasingly block the response to 2-sec application of 30 μM nicotine. *Right*, Dose-response curves for several antagonists are shown normalized to the initial response to 30 μM nicotine. The average ± standard error of three to five experiments at −60 mV are shown. The *lines* are fits of the Hill equation to the data.

by cloning of a full length α7 cDNA from a library prepared from these cells. A 2.1-kb α7 cDNA was identified through screening of a λZap II (Stratagene) library, using as a probe rat α7 cDNA (provided by Drs. Jim Boulter and Steve Heinemann at The Salk Institute). The nucleotide sequence is shown in Fig. 2. Northern blot analysis using this cDNA as a probe revealed that SH-SY5Y cells express mRNAs of three sizes (5.9 kb, 2.6 kb, and 1.3 kb), which hybridized to this probe at high stringency (data not shown). This SH-SY5Y cDNA library may prove to be a good source for cloning structural subunits that may be associated with α7 subunits in native α7 AChRs, when suitable probes become available.

The mature $\alpha 7$ subunit peptide sequence is highly conserved, showing 94% identity to rat and 92% identity to chicken $\alpha 7$ sequences (Fig. 3). There is very limited sequence diversity in the putative large extracellular domain amino-terminal of the first putative transmembrane sequence, which is thought to be primarily responsible for forming the ACh binding site (15, 36) and sites for specific interactions between adjacent subunits (37). This suggests that human $\alpha 7$, like chicken and rat $\alpha 7$ (6, 7, 20), should efficiently assemble into functional homomers. Extensive amino-terminal sequence identity also implies that there are very few sequence differences to account for pharmacological differences between species. The putative transmembrane sequences M1, M2, and M3 are identical in all three species, implying that if, as expected, the M2 sequence is primarily responsible for lining the cation channel (3, 38) then the cation channel properties of human $\alpha 7$ homomers should be identical to those of homomers of $\alpha 7$ from chickens and rats (6, 7, 22). The most sequence diversity is in the large putative cytoplasmic domain between putative transmembrane sequences M3 and M4, as is typical for interspecies sequence variation of AChR subunits.

The epitope for mAb 306, which cross-reacts best between chicken and human $\alpha 7$, has been mapped, using synthetic peptides, to within the sequence 380–400 (30). Several other mAbs to this region did not cross-react, and so one or more of the five amino acids that differ between chick and human $\alpha 7$ in this sequence must be important for their binding, whereas mAb 306 may have its epitope centered on one of the three nearly contiguous stretches of four identical amino acids between residues 380 and 393. mAb 319 binds to an epitope within the sequence 365–384 (30). Within this sequence only three amino acids differ between chick and human $\alpha 7$, with 11 consecutive identical amino acids between residues 365 and 375 that might contain the epitope.

Human $\alpha 7$ was expressed in *Xenopus* oocytes by injecting saturating amounts (100 ng) of mRNA into each oocyte. This resulted in efficient assembly of $\alpha 7$ subunits into homomers and expression on the cell surface of ~63% of the ~3–4 fmol/oocyte of total ^{125}I-αBgt binding sites typically observed. This high level of surface expression is comparable to the level observed with the native subunit compositions $\alpha 1\beta\gamma\delta$ and $\alpha 4\beta 2$ expressed in oocytes (39), is typical of that for $\alpha 7$ homomers from chicken (20), and is much higher than that for $\alpha 8$ homomers from chicken (22).

Pharmacological properties of native $\alpha 7$ AChRs solubilized from SH-SY5Y cells and of human $\alpha 7$ homomers solubilized from *Xenopus* oocytes were studied using mAb 306 attached to microwells to immobilize these proteins. The affinity of ^{125}I-αBgt binding to native $\alpha 7$ AChRs measured in this way (K_d = 1.06 nM) (see Fig. 4) was similar to that reported using SH-SY5Y membrane fragments (K_d = 4 nM) (24) and close to that observed for solubilized native chicken $\alpha 7$ AChRs in the same solid-phase assay using mAb 318 to the same epitope (K_d = 1.91 nM) (20) (see Table 1). The affinity of human $\alpha 7$ homomers for ^{125}I-αBgt (K_d = 0.81 nM) (Fig. 4) was nearly identical to that of native human $\alpha 7$ AChRs, as is typical of chicken $\alpha 7$ homomers (20) (Table 1). The level of expression of $\alpha 7$ AChRs in SH-SY5Y cells is not especially high, and it is interesting to note that a single oocyte can produce as many $\alpha 7$ ^{125}I-αBgt binding sites as can 3×10^5 SH-SY5Y cells.

Binding of small ligands was measured by inhibition of ^{125}I-αBgt binding, as shown by examples in Fig. 5. The results of all binding studies conducted are compared in Table 1 with similar data for chicken native $\alpha 7$ AChRs and $\alpha 7$ homomers reported by Anand *et al.* (20, 21), when mAb 318 to the same sequence region recognized by mAb 306 (30) was used to immobilize chicken $\alpha 7$ AChRs and $\alpha 7$ homomers. Human $\alpha 7$ AChRs and $\alpha 7$ homomers exhibit moderate affinity for cholinergic agonists, as might be expected for detergent-solubilized AChRs, which are probably in a desensitized conformation. Despite the extensive sequence identity of $\alpha 7$ between species, there are substantial differences between species for some ligands. The classical nicotinic antagonist curare does not have especially high affinity for $\alpha 7$ AChRs. Remarkably, the classical glycinergic antagonist strychnine has equal or greater affinity, compared with curare. The affinity of the classical muscarinic AChR antagonist atropine is much lower for human $\alpha 7$ than it is for chicken $\alpha 7$.

The pharmacological properties of the ACh binding site revealed by the ligands studied in Table 1 suggest that the ligands can be resolved into two groups, 1) those that bind similarly in both species (e.g., cytisine, nicotine, and curare) and 2) those that show large species-specific differences in binding (e.g., ACh, carbamylcholine, tetramethylammonium, and atropine). In both cases the pharmacological properties of the native $\alpha 7$ AChRs reflect properties of the $\alpha 7$ homomers. Defining the pharmacological properties of native human $\alpha 7$ AChRs and human $\alpha 7$ homomers will be important for evaluating success in reconstituting the native subunit composition in *Xenopus* oocytes if putative structural subunits for $\alpha 7$ AChRs are cloned.

Human $\alpha 7$ homomers expressed in *Xenopus* oocytes exhibited strong, rapidly desensitizing responses to agonists (Fig. 6). The maximum response to 100 μM nicotine in cells held at −70 mV was about 10 μA. Currents showed extremely strong inward rectification, with a reversal potential of -15 ± 2 mV (Fig. 6). These properties are also typical of chicken $\alpha 7$ and $\alpha 8$ homomers (6, 22) and rat $\alpha 7$ homomers (7).

Activation of human $\alpha 7$ homomers, like $\alpha 7$ and $\alpha 8$ homomers from chickens and rats, results in influx of Ca^{2+}, which triggers an endogenous Ca^{2+}-dependent Cl^- current (7, 16, 22). Fig. 7 illustrates separation of the cation current through the $\alpha 7$ homomer channel from the secondary anion current. In Fig. 7A the holding potential was clamped at −13 mV, which is between the reversal potential of the primary $\alpha 7$-mediated current at ≈0 mV and the reversal potential of the secondary Ca^{2+}-triggered Cl^- current at approximately −18 mV. In normal saline solution (1.8 mM Ca^{2+}) the response was biphasic, with a fast initial current through $\alpha 7$ homomers followed by a prolonged outward current through Cl^- channels, which inactivate more slowly. In saline solution lacking Ca^{2+} only the inward current through the $\alpha 7$ homomer remained. Shifting the holding potential to −20 mV, as shown in Fig. 7B, changed the direction of the Ca^{2+}-dependent current flow to inward. At the typical holding potential of −70 mV the $\alpha 7$ response in Ca^{2+}-free saline solution was only 20% of the response in normal saline solution.

The rapid desensitization, Ca^{2+} permeability, and inward rectification exhibited by human $\alpha 7$ homomers are typical of chicken and rat $\alpha 7$ subunit homomers and chicken $\alpha 8$ homomers (7, 22, 26). This is expected, because the M2 transmem-

brane domain sequence thought to be primarily responsible for forming the cation channel is identical in all cases.

Responses to several agonists are shown in Fig. 8. For several ligands such as ACh and nicotine these are typical of chicken and rat α7 homomer responses (7, 17, 22). Agonists were half-maximally effective at concentrations 4–45-fold higher than their half-maximal equilibrium binding concentrations, and potency reflected binding affinity. Agonists exhibited Hill coefficients of ≈2, as is typical of nicotinic AChRs, which are expected to require liganding of two binding sites for activation and then relax into a desensitized state with higher affinity for agonists (40). However, there are some significant species-specific differences in potency and efficacy (Table 2). For example, nicotine and cytisine are 4–5-fold less potent with human α7 homomers than with chick α7 homomers. A striking difference in efficacy is shown by DMPP, which is only a weak partial agonist with chicken α7 homomers (giving <3% of the maximum response of full agonists) but is a full agonist with human α7 homomers. Tetramethylammonium is nearly as potent as ACh, and DMPP is even more potent than ACh. These dose-response studies were conducted with the oocytes held at −60 mV because it was previously shown that for chick α7 and α8 homomers at this voltage the secondary Ca^{2+}-dependent Cl^- current appeared to be proportional to the activation (22, 26).

Antagonist effects are shown in Fig. 9. Small molecule antagonists were typically half-maximally active at concentrations 12–46-fold lower than their half-maximal equilibrium binding concentrations. These antagonists exhibited Hill coefficients of ≈1, as would be expected if antagonist binding to only one of the (perhaps) five binding sites in a homomer could prevent the conformational change to the active state. Human α7 homomers were 3.5–14-fold less sensitive to all of the antagonists tested than were chicken α7 homomers (Table 2).

Despite the extremely conserved amino-terminal putative extracellular domain, which is thought to contain all of the amino acids forming the ACh binding sites in α7 homomers (all but 11 of these 207 amino acids are identical in chicken and human α7), there are substantial pharmacological differences between human and chick α7 homomers (Figs. 4, 5, 8, and 9; Tables 1 and 2). Those amino acids that differ do not include any of the amino acids implicated in formation of the ACh binding site in α1, by photoaffinity labeling (36), by affinity labeling (3), or by mutagenesis in α7 (15), nor are they homologues of δ subunit residues identified by labeling and mutagenesis (41). Thus, investigation of the residues that account for these pharmacological differences may provide some new insights into sequences that contribute to formation of the ACh binding site.

The structural, pharmacological, and functional properties of α7 AChRs from different species are just beginning to be defined, and determining their normal functional roles and pathological significance remains a challenge for the future.

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Send reprint requests to: Jon Lindstrom, 217 Stemmler Hall, Department of Neuroscience, University of Pennsylvania Medical School, Philadelphia, PA 19104–6074.