Homomeric and native α7 acetylcholine receptors exhibit remarkably similar but non-identical pharmacological properties, suggesting that the native receptor is a heteromeric protein complex

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Sucrose gradient analysis of chick acetylcholine receptor (AChR) α7 subunits expressed in oocytes indicates that they form pharmacologically active homomers of the same size as native α7 AChRs, a size compatible with a complex of five α7 subunits. By immunisolating the [35S]methionine-labeled α7 subunits we also demonstrate that they do not appear to assemble with endogenous Xenopus AChR subunits. Pharmacological characterization of detergent-solubilized brain α7 AChRs and α7 homomers reveals that they have similar but nonidentical properties. The pharmacological difference is most accentuated for cytisine (~50-fold). Thus, at least in F18 chicken brain, most or all of the native α7 AChRs do not appear to be homomeric.

1. INTRODUCTION

α-Bungarotoxin (αBgt) is a potent antagonist of the well characterized muscle-type acetylcholine receptors (AChRs). However, αBgt also antagonizes a subset of neuronal AChRs, of which some subunits (α7 and α8) have been cloned [1,2]. The α7 [2] and α8 subunits (Gerzanich et al., in preparation) form functional homomers when expressed in oocytes. The electrophysiological properties of the homomers formed by expressing normal and mutated α7 subunits in oocytes have been the target of many recent investigations [3–7].

The subunit composition and function of the native neuronal AChRs which bind αBgt are just starting to be dissected. Despite the long standing knowledge that neuronal αBgt-sensitive proteins display nicotinic character (reviewed in [8,9]), a more detailed characterization of these AChRs was made possible using α7 and α8 subunit-specific mAbs. Using these mAbs it has been shown that neuronal AChRs which bind αBgt are composed of at least three subtypes i.e. those containing α7 subunits (α7 AChRs), α8 subunits (α8 AChRs), and both α7 and α8 subunits (α7/α8 AChRs) [1,10–12]. More recently, the contrasting pharmacological properties of the α7 AChRs and α8 AChRs immunisolated from retina have been characterized (Anand et al., manuscript submitted). At the protein level, while the results of numerous attempts to affinity purify these AChRs all show multiple bands on SDS-acrylamide gel electrophoresis, indicating that they are likely to be made up of more than one subunit [13–17], there has not been an unambiguous demonstration of the total number of subunits or the stoichiometry in which these subunits associate to form the various native neuronal AChR subtypes which bind αBgt. At the functional level, preliminary characterization of nicotine-induced, αBgt-sensitive currents (presumably of α7 AChRs) from cultured rat hippocampal neurons have been reported [18–20].

In this paper, we characterize both native brain α7 AChRs and α7 homomers expressed in Xenopus oocytes. Although exogenous AChR subunits translated in oocytes have been reported to assemble with endogenous Xenopus AChR subunits [21], we demonstrate that no additional proteins recognizable as additional bands on SDS-acrylamide gel electrophoresis are associated with α7 subunits expressed in Xenopus oocytes. We also show that the size of the α7 homomers is compatible with a complex of five α7 subunits. Furthermore, we characterize in detail the pharmacological properties of both the α7 homomers and the immunisolated native α7 AChR subtype from chick brain. Comparison of their pharmacological properties reveals that they exhibit remarkable similarities in their affini-
ties for many ligands. However, differences in affinities for some ligands indicate that the native α7 AChR is most likely to be composed of more than one kind of subunit.

2. MATERIALS AND METHODS

2.1. mAbs

mAbs to α7 (318) and to α8 (305) have been previously described [1]. The epitope for mAb 318 was mapped using synthetic peptides to within α7 380-400 [22], thus all α7 AChR and α7 homomer ligand binding studies reported here were done with subunits tethered through their putative large cytoplasmic domain, an interaction which is unlikely to alter properties of the ACh binding site on their extracellular surface. mAb 305 binding was found to depend on the native conformation of α8 [22]. The mAbs were affinity purified using protein G agarose.

2.2. Expression of α7 homomers in oocytes

The α7 cDNA was cloned into a modified SP64T expression vector [23] using standard DNA cloning procedures. In vitro RNA was synthesized using a standard protocol [23] and more recently using the Megascript kit (Ambion, Austin, TX). Oocytes were prepared for injections as described in [24] and injected with ~15 ng of cRNA per oocyte. The oocytes were incubated in semi-sterile conditions at 18°C for 3–4 days before use. Metabolic labeling of expressed α7 subunits was achieved by incubating injected oocytes in saline solution containing 0.5 mM [35S]methionine (~1,000 Ci/mmol, Amersham) for 3.4 days.

2.3. Purification and solubilization of AChRs from oocytes and chicken brain

Oocytes expressing homomeric α7 AChRs were homogenized in lysis buffer (2% Triton X-100, 50 mM NaCl, 50 mM sodium phosphate (pH 7.5), 5 mM EDTA, 5 mM EGTA, 2 mM phenylmethylsulfonyl fluoride, 5 mM benzamidine, 5 mM iodoacetamide, 1 mg/ml heat-denatured BSA) incubated at 4°C for 30 min, and centrifuged for 10 min in a microfuge to clear the cellular debris. The cleared lysate was then used for all the assays. The preparation of Triton X-100-solubilized native neuronal AChRs which bind αSGt and the method of immunosolation of the native α7 AChR subtype from these extracts have previously been described [12]. Briefly, Triton X-100 extracts from brain were depleted of the α7/α8 and α8 AChR subtypes by an overnight incubation of ~7 ml of brain extract with ~1 ml of mAb 305-coupled Actigel beads (Sterogene, 5 mg/ml gel). The beads were then removed by brief centrifugation. The extent of depletion was then tested by solid-phase RIAs using microwells coated with mAb 305 in the presence of 50 nM [35S]methionine per fraction determined by liquid scintillation counting. The inset panel shows a fluorogram of the [35S]methionine-labeled protein immunoimmobilized on the wells the immunoimmobilized protein was measured using 50 nM [35S]methionine (1.000 Ci/mmol, Amersham) for 334 days.

2.4. Sucrose gradient sedimentation analysis

Aliquots (~300 μl) of extracts from either chick brain or ~10 oocytes were layered on to 11 ml sucrose gradients (5–20%) as previously described [25], and the sedimentation of AChR analyzed by [35S]methionine-labeled α7 subunits on sucrose gradients were eluted off in sample buffer and electrophoresed on 10% SDS-polyacrylamide gels. The gels were then treated for fluorography, dried and exposed to X-ray films. The gel slices containing the α7 subunit excised, and the amount of radioactivity in the gel slice determined by liquid scintillation counting.

2.5. Pharmacological assays

Pharmacological characterization of the expressed and immunosolated brain α7 AChRs was performed by competitive inhibition of [35S]αSGt binding by various ligands to AChRs immunomobilized on mAb 318-coated Immulon 4 microtiter plates. Triton X-100 extracts were added to each well in the presence of various concentrations of the ligands and incubated for 20 min prior to the addition of [35S]αSGt. The assays in duplicate were performed in the presence of 2 nM [35S]αSGt in a total volume of 100 μl. After incubation overnight at 4°C, the wells were rinsed three times with ~250 μl of PBS/Tween 20 buffer and then counted in a γ counter. The affinity of αSGt for the AChRs was also measured by similar solid-phase RIAs except that increasing amounts of [35S]αSGt were used. Non-specific binding in all cases was determined using wells not coated with mAbs.

3. RESULTS

3.1. Immunological evidence that expressed α7 subunits form homomers

Using sucrose gradient sedimentation analysis of α7 subunits expressed in oocytes, we show that [35S]methionine-labeled α7 subunits bound activity of both expressed α7 subunits and native brain α7 AChRs cosediment at ~10 S (Fig. 1). This
Fig. 2. Binding of [125I]Bgt to detergent-solubilized brain α7 AChRs and α7 homomers. Binding of [125I]Bgt to Triton X-100 solubilized AChRs was performed on AChRs immobilized on Immulon 4 microwells coated with a mAb to α7. Non-specific binding was determined using wells not coated with the mAb. Each data point is the mean of the values obtained from duplicate determinations. The insert shows Scatchard analysis of the data, displayed as bound/free (pmol/pmol) vs. bound (pmol/l). The symbols at the right top corner of each plot shows the putative subunit composition of the α7 AChRs, assuming that the AChRs are pentameric and that the native α7 AChRs have only two α7 ligand-binding subunits per AChR, as do (α1)βγδ and (α4)2(β2), AChRs [25,26].

indicates that native α7 AChRs and α7 homomers have the same basic size and shapes. Native α7 AChRs and functional α7 homomers might be expected to exhibit the pentagonal symmetry of other members of the AChR gene family such as muscle αβγδ AChR or neuronal (α4)2(β2), AChR [25,26]. The calculated protein molecular weight of the Torpedo αβγδ AChR monomer is 267,757 Da. In comparison, the calculated protein molecular weight of the chicken α7 pentamer is 272,750 Da. Thus α7 pentamers would sediment somewhat faster than the Torpedo αβγδ AChR monomer, as is observed.

Remarkably, metabolic labeling of the expressed α7 protein with [35S]methionine indicates that these subunits form a broad array of protein complexes of which only the ones assembled into the size of native AChRs are capable of binding [125I]Bgt. Immunolocalization of the ~10 S [35S]methionine-labeled protein complex using a mAb to α7, followed by fractionation of this complex by SDS-PAGE reveals that it is made up of only one band of apparent molecular weight of ~60 kDa (shown in the inset panel of Fig. 1). It has been reported that muscle AChR subunits expressed in oocytes from cRNA could form small amounts of functional AChRs by assembling with AChR subunits endogenously expressed in Xenopus oocytes [21], but unless the oocytes contain an endogenous subunit of the same apparent molecular weight as α7, these data argue that α7 subunits expressed in oocytes do not appear to assemble with other endogenously expressed subunits of the Xenopus AChR, but instead form homomers the size of native AChRs.

3.2. Immunolocalization of native α7 AChRs from detergent-solubilized chick brain extracts

We have previously shown that in E18 chick brain ~68% of all αBgt-sensitive AChRs are of the α7 AChR subtype, ~9% are of the α8 AChR subtype, and ~23% are of the mixed α7/α8 AChR subtype [12]. To obtain extracts containing only α7 AChRs, we depleted Triton X-100 extracts of brain with a mAb to α8 coupled to agarose beads using an extract to mAb-bead ratio of 7:1 (v/v). The efficacy of depletion was then tested by solid phase RIAs using mAb microwells coated with a mAb to α8. Thus, we typically achieved >99% depletion of all α8-containing AChRs (including the α8 AChRs and the α7/α8 AChRs). These depleted extracts were then used for all pharmacological assays.

3.3. Scatchard analysis of [125I]αBgt binding to immunolocalized brain α7 AChRs and α7 homomers

Saturation binding curves shown in Fig. 2 were achieved by incubating increasing amounts [125I]αBgt with native α7 AChRs and α7 homomers immunolocalized on microwells. Scatchard analysis of these binding curves reveals that α7 homomers bind [125I]αBgt with a high affinity (Kd = 1.62 ± 0.08 nM) that is nearly identical to that of the native α7 AChR subtype from chick brain (Kd = 1.66 ± 0.04 nM).

3.4. Pharmacological characterization of native α7 AChRs and α7 homomers

Further pharmacological characterization to test the relative efficacies by which various cholinergic ligands and some atypical ligands, such as strychnine and atropine, inhibit [125I]αBgt binding to these AChRs was carried out using solid-phase RIAs. Fig. 3 shows the [125I]αBgt inhibition curves for a subset of all the ligands tested. The calculated Kᵢ’s for all the ligands tested are shown in Table I. Thus the relative affinities of the various ligands for the α7 homomers was found to be αBgt > > cytisine > > nicotine > curare ≈ strychnine > > trimethaphan > > ACh > TMA > > atropine > > Carb > Deca > TEA > Hexa. The affinities of the ligands for the native brain α7 AChRs, however, were found to be
\(\alpha\text{Bgt} > \) nicotine \(\approx\) curare \(\approx\) cytisine \(\approx\) strychnine > trimethaphan > ACh > atropine \(\approx\) TMA > Deca > Carb > Hexa > TEA. While many ligands showed smaller differences in affinity for the \(\alpha 7\) homomers compared to the native \(\alpha 7\) AChR, cytisine appeared to be the most discriminatory, showing nearly a 50-fold difference in affinity (Fig. 4).

Interestingly, both the native \(\alpha 7\) AChR and \(\alpha 7\) homomers appeared to be sensitive to strychnine, a glicinenergic antagonist, and to atropine, a muscarinic antagonist. It does not appear that strychnine or atropine have remarkably high affinity, but that several classic cholinoergic ligands (e.g. curare or Carb) do not have remarkably greater affinity, or even lower affinity (ACh). Even the simplest of possible ligands, TMA, has substantial affinity as compared to curare, carbamylcholine, atropine, and strychnine. It is interesting to note that strychnine has been reported to inhibit the function of rat \(\alpha 7\) homomers [7] and of \(\alpha\text{Bgt}\)-binding AChRs on chick cochlear hair cells [27].

4. DISCUSSION

In this study we first demonstrate that \(\alpha 7\) subunits expressed in Xenopus oocytes form \(\alpha\text{Bgt}\)-binding \(\alpha 7\) homomers that co-sediment with native brain \(\alpha 7\) AChRs on sucrose gradients. The size of the homomeric AChRs is compatible with a pentamer of \(\alpha 7\) subunits. Using \(^{13}S\)methionine to metabolically label the \(\alpha 7\) subunits, we also show that the \(\alpha 7\) subunits do not appear to assemble with endogenously expressed Xenopus AChR subunits. The observation that binding of \(^{125}\)I\(\alpha\text{Bgt}\) only occurs when \(\alpha 7\) subunits assemble in complexes of the size of native \(\alpha 7\) AChRs suggests that the ability to bind both \(\alpha\text{Bgt}\) and small cholinoergic ligands is acquired only after assembly of the \(\alpha 7\) subunit into complexes of native stoichiometry, perhaps because the binding sites are formed by distinct parts of adjacent subunits. This contrasts with the case of muscle \(\alpha 1\) subunits which can bind \(\alpha\text{Bgt}\) but acquire affinity for small cholinoergic ligands only after pairing up with either the \(\gamma\) or \(\delta\) subunits [28,29].

The native neuronal AChRs which bind \(\alpha\text{Bgt}\) have evaded detailed characterization since they were first detected. Here, using \(\alpha 7\) and \(\alpha 8\) subunit-specific mAbs, we isolated the detergent-solubilized \(\alpha 7\) AChR subtype from chick brain extracts. Then by characterizing the pharmacological properties of mAb-tethered, detergent-solubilized \(\alpha 7\) homomers and brain \(\alpha 7\) AChRs under the same assay conditions, we were able to make a meaningful comparison of their pharmacological properties for the first time.

A striking feature of this comparison is that the pharmacological properties of the \(\alpha 7\) homomers are very similar to those of native \(\alpha 7\) AChRs. However, the \(\alpha 7\) homomers show a \(\sim\) 50-fold higher affinity for binding cytisine than do the brain \(\alpha 7\) AChRs. This feature, in addition to the smaller differences observed with some of the other ligands, suggests that most if not all of the native \(\alpha 7\) AChRs expressed in E18 chick brain do not exist as homomers, although it is conceivable that these \(\alpha 7\) subunits might assemble into homomers at other times during development. While we cannot incontrovertibly exclude the possibility that some of the pharmacological differences observed are due to post-translational modifications of the \(\alpha 7\) AChR expressed in oocytes, this appears unlikely to be the case because at least for Torpedo AChRs expressed in oocytes, despite altered patterns of N-linked glycosylation of the subunits [30], these AChRs were pharmacologically and functionally indistinguishable from the native Torpedo AChRs [31,32]. All published accounts of purified vertebrate brain \(\alpha\text{Bgt}\) binding AChRs report more than one band on SDS acrylamide gel electrophoresis [13–17], although in no case has it been proven that these components are additional AChR subunits. The pharmacological data shown here strongly support the notion that additional subunits co-assemble with the \(\alpha 7\) subunits in the native AChR. The concentrations at which cytisine activates has previously been shown to be very sensitive to the type of structural subunits present. For example, co-expression of \(\alpha 3\) and \(\beta 2\) subunits result in a \(\sim\) 100-fold greater efficacy for cytisine as compared to co-expression of \(\alpha 3\) and \(\beta 4\) subunits [33]. Thus while structural subunits do participate in the binding of, or indirectly influence the binding of, \(\alpha 7\) subunits to at least some of the ligands, they appear to have a limited influence on the binding of many of the ligands by the native \(\alpha 7\) AChR.

The neuronal \(\alpha 7\) AChRs (the predominant brain \(\alpha\text{Bgt}\)-sensitive AChR subtype) exhibit several different pharmacological properties from the neuronal \(\alpha 4\beta 7\) AChRs (the predominant brain \(\alpha\text{Bgt}\)-insensitive sub-

### Table I

**Pharmacological characterization of \(\alpha 7\) AChRs**

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Affinity ((K_i, \mu M))</th>
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<tbody>
<tr>
<td>Homomeric</td>
<td>Brain</td>
</tr>
<tr>
<td>Acetylcholine</td>
<td>24.9 ± 4.7</td>
</tr>
<tr>
<td>Atropine</td>
<td>148 ± 52</td>
</tr>
<tr>
<td>2-Bungarotoxin</td>
<td>0.00162 ± 0.00008</td>
</tr>
<tr>
<td>Carbamylcholine</td>
<td>250 ± 11</td>
</tr>
<tr>
<td>Curare</td>
<td>4.97 ± 0.5</td>
</tr>
<tr>
<td>Cytisine</td>
<td>0.0775 ± 0.001</td>
</tr>
<tr>
<td>Decamethonium</td>
<td>376 ± 36</td>
</tr>
<tr>
<td>Hexamethonium</td>
<td>891 ± 174</td>
</tr>
<tr>
<td>l-Nicotine</td>
<td>0.545 ± 0.02</td>
</tr>
<tr>
<td>Strychnine</td>
<td>6.85 ± 0.44</td>
</tr>
<tr>
<td>Tetraethylammonium</td>
<td>521 ± 64</td>
</tr>
<tr>
<td>Tetramethylammonium</td>
<td>999 ± 1.9</td>
</tr>
<tr>
<td>Trimethaphan</td>
<td>18.0 ± 3.0</td>
</tr>
</tbody>
</table>
We find that α7 AChRs show less than 10-fold lower affinity for antagonists like curare and hexamethonium, but 600- to 27,000-fold lower affinity for agonists such as nicotine, carbamylcholine, or cytisine, when compared to the affinities of the α4β2 AChRs [34]. Another interesting feature of α7 homomers is that they show only a small difference in apparent affinity for the agonist, nicotine, between their activatable state (EC₅₀ ~ 7.8 μM, Gerzanich et al., manuscript in preparation) and their presumably desensitized state (Kᵦ ~ 0.5 μM). This contrasts with α4β2 AChRs, which exhibit a large difference in their apparent affinity for nicotine between the two states with an EC₅₀ of ~1 μM, for the activatable state, and a Kᵦ of ~3.9 nM [34], for the presumably desensitized state. At the functional level, it is interesting and perhaps toxicologically important to note the equipotency of nicotine and ACh (~1 μM) for α4β2 AChRs expressed in oocytes [35,36] compared to the 14:1 potency ratio of nicotine vs. ACh seen for α7 homomers [2]; Gerzanich et al., manuscript in preparation), and the even greater (72-fold) selectivity of native α7 AChRs for nicotine vs. ACh (Table I).

Cloning of AChR subunit cDNAs has expanded the repertoire of AChR subtypes that might be expressed in the central and peripheral nervous system. However, thus far biochemical techniques have not uniquely identified structural subunits in α7 AChRs and no candidate cDNAs have been identified for structural subunits for β-gt-binding AChRs. In this study, by comparing and contrasting the properties of the native α7 AChRs and α7 homomers, we have provided pharmacological tools which will help authenticate the cloning of structural subunit cDNAs which should then allow the reconstitution and study of native α7 AChRs in expression systems devoid of other AChRs.

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![Graph](https://example.com/graph.png)

**Fig. 3.** Pharmacological characterization of detergent-solubilized brain α7 AChRs and α7 homomers. Pharmacological characterization was performed by competitive inhibition of [125I]β-gt binding by various ligands to AChRs solubilized with Triton X-100 and then immobilized on mAb 312-coated Immunon 4 microwells. Competitive inhibition was performed in the presence of 2 nM [125I]β-gt. Representative dose–response curves from one experiment are shown. Each data point is the mean of the values obtained from duplicate determinations.
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