Assembly of *Torpedo* Acetylcholine Receptors in *Xenopus* Oocytes

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Abstract. To study pathways by which acetylcholine receptor (AChR) subunits might assemble, *Torpedo* α subunits were expressed in *Xenopus* oocytes alone or in combination with β, γ, or δ subunits. The maturation of the conformation of the main immunogenic region (MIR) on α subunits was measured by binding of mAbs and the maturation of the conformation of the AChR binding site on α subunits was measured by binding of α-bungarotoxin (αBgt) and cholinergic ligands. The size of subunits and subunit complexes was assayed by sedimentation on sucrose gradients. It is generally accepted that native AChRs have the subunit composition $\alpha_2\beta\gamma\delta$. *Torpedo* α subunits expressed alone resulted in an amorphous range of complexes with little affinity for αBgt or mAbs to the MIR, rather than in a unique 5S monomeric assembly intermediate species. A previously recognized temperature-dependent failure in α subunit maturation may cause instability of the monomeric assembly intermediate and accumulation of aggregated denatured α subunits. Coexpression of α with β subunits also resulted in an amorphous range of complexes. However, coexpression of α subunits with γ or δ subunits resulted in the efficient formation of 6.5S $\alpha\gamma$ or $\alpha\delta$ complexes with high affinity for mAbs to the MIR, αBgt, and small cholinergic ligands. These $\alpha\gamma$ and $\alpha\delta$ subunit pairs may represent normal assembly intermediates in which *Torpedo* α is stabilized and matured in conformation. Coexpression of α, γ, and δ efficiently formed 8.8S complexes, whereas complexes containing $\alpha\beta$ and γ or $\alpha\beta$ and δ subunits are formed less efficiently. Assembly of β subunits with complexes containing $\alpha\gamma$ and δ subunits may normally be a rate-limiting step in assembly of AChRs.

THE nicotinic acetylcholine receptor (AChR)[1] from muscle and *Torpedo* electric organ is a membrane protein composed of four homologous subunits with the stoichiometry $\alpha_2\beta\gamma\delta$ (reviewed in Karlin et al., 1986; McCarthy et al., 1986; and Maelicke, 1988; Changeux, 1990). It is generally agreed that the subunits are organized like barrel staves around a central cation channel, but there is conflicting evidence indicating that the order of the subunits is either $\alpha\beta\alpha\gamma\delta$ (Kubalek et al., 1987; Unwin, 1989) or $\alpha\gamma\alpha\beta\delta$, or $\alpha\delta\alpha\beta\gamma$ (Karlin et al., 1983; Blount and Merlie, 1989; Pedersen and Cohen, 1990). The acetylcholine (ACh) binding site regulates opening of the cation channel. Some of the amino acids that contribute to its structure are located near disulfide-linked cysteines 192 and 193 on the AChR α subunit (Kao et al., 1984; Kao and Karlin, 1986; Pedersen et al., 1986; Neumann et al., 1986; Dennis et al., 1988; Abramson et al., 1989). Other agonists and antagonists such as carbamylcholine, curare, and α-bungarotoxin (αBgt) are thought to also bind to the same site. The main immunogenic region (MIR), like the AChR binding site, is located on the extracellular surface of AChR α subunits, but amino acids within α68–76 are critical to its structure (Barkas et al., 1988; Bellone et al., 1989; Tzartos et al., 1990; Das and Lindstrom, 1989; Saedi et al., 1990). This region is the site of binding for the majority of the autoantibodies produced in myasthenia gravis patients (Tzartos et al., 1982) and in animals with experimental autoimmune myasthenia gravis (Tzartos et al., 1981).

AChR α subunits, when initially synthesized, do not bind αBgt or small ligands with high affinity (Merlie and Lindstrom, 1983; Carlin et al., 1986). By 15–30 min after synthesis, before associating with other AChR subunits, a fraction of AChR α subunits undergo conformational maturation to assembly intermediates and acquire the ability to bind αBgt with moderate affinity, but they still lack the ability to bind small cholinergic ligands. Expressing mouse AChR α subunits alone in fibroblasts (Blount and Merlie, 1988) or *Torpedo* AChR α subunits in *Xenopus* oocytes (Kurosaki et al., 1987; Sumikawa and Miledi, 1989) also resulted in only a small fraction of α subunits that can bind αBgt but not small ligands. Human TE671 cells produce large numbers of monomeric α subunits with the conformation of assembly intermediates (Conroy et al., 1990). Expressing AChR α subunits in combination with the γ or δ, but not β, subunits in *Xenopus* oocytes (Kurosaki et al., 1987; Sumikawa and

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1. *Abbreviations used in this paper*: αBgt, α-bungarotoxin; AChR, acetylcholine receptor; MIR, main immunogenic region.

Miledi, 1989) or fibroblasts (Blount and Merlie, 1989) resulted in substantially increased affinity of the AChR α subunit for small ligands. These findings indicate that association of AChR α with γ or δ, but not β, is a prerequisite for the binding of small ligands and suggest that the α subunit undergoes substantial conformational maturation at the ACh binding site during the assembly of AChR when it associates with the γ or δ subunit (Blount and Merlie, 1989), and/or that γ or δ subunits contribute amino acids to the ACh binding site (Pedersen and Cohen, 1990). The two ACh binding sites on AChR molecules are not identical. This was shown by the differences in the affinity of the two α subunits on native AChR for curare (Hamilton et al., 1985; Neubig and Cohen, 1979; Ratnam et al., 1986*a*), affinity labeling reagents (Damle and Karlin, 1978; Ratnam et al., 1986*a*), and mAbs (Whiting et al., 1985; Fels et al., 1986; Mihovilovic and Richman, 1987; Dowding and Hall, 1987). Recent studies from pair-wise expression of mouse muscle AChR subunits in quail fibroblasts (Blount and Merlie, 1989) and from using ^{3}H-curare as an affinity-labeling reagent on native *Torpedo* AChR (Pedersen and Cohen, 1990) indicate that association of AChR α subunits with γ subunits is required for the high-affinity binding of curare, whereas association of AChR α subunits with δ subunits is required for the low-affinity binding of curare.

The conformation change of other regions on the AChR α subunit during AChR assembly has not been extensively studied. Merlie and Lindstrom (1983) showed that in the mouse BC3H-1 cell line, AChR α subunits acquire the ability to bind mAb 35, a conformationally dependent, MIR-specific mAb (Das and Lindstrom, 1989; Tzartos et al., 1988), concurrent with maturing to assembly intermediates and acquiring the ability to bind αBgt with moderate affinity. We also showed that in the human TE671 cell line, unassembled monomeric (5.0S) AChR α subunits with moderate affinity for mAb 35 are synthesized, but that this affinity is substantially lower than the affinity of native TE671 AChR for this mAb (Conroy et al., 1990). *Torpedo* AChR α subunits expressed in mammalian fibroblasts apparently tend to denature and lose affinity for αBgt and mAb 35 unless they are stabilized by association with other subunits (Paulson and Claudio, 1990).

Xenopus oocytes have proven to be an efficient system for expressing functional AChRs when injected with *Torpedo* electric organ mRNA (Sumikawa et al., 1981), or when injected with in vitro-synthesized AChR subunit mRNA (Mishina et al., 1985; White et al., 1985). Due to this efficiency, expressing different combinations of AChR subunits in *Xenopus* oocytes has been used to address some questions regarding the assembly of AChRs. It was shown that fully functional AChR molecules are efficiently formed when all four AChR subunits are expressed, but that very small but electrophysiologically detectable amounts of partially functional complexes are also formed in the absence of β, γ, or δ subunits (Kullberg et al., 1990; Kurosaki et al., 1987; Mishina et al., 1984; White et al., 1985). Recently, immunoprecipitation of oocyte extracts was used to show that the association of AChR subunits still occurred in the absence of one or two of the other AChR subunits, but that efficient insertion of AChR into the surface membranes required all four subunits (Sumikawa and Miledi, 1989). These results indicate that AChR subunits associate and form some incomplete AChR molecules in *Xenopus* oocytes. However, they do not show whether these associations form specific or unique complexes and if so, what are the sizes or compositions of these complexes.

In the present study, the expression of *Torpedo* AChR α subunits in combination with various other AChR subunits in *Xenopus* oocytes was studied to analyze the conformation change in the α subunit upon association with other AChR subunits, and to identify the subunit composition and size of the complexes that may be specifically and efficiently formed during the assembly of AChR subunits. *Torpedo* α subunits expressed alone, unlike mammalian α subunits, were not stable in the form of 5S assembly intermediates, but formed denatured aggregates. Coexpression of α and β subunits did not prevent denaturation of α subunits. Coexpression of α with γ or δ subunits in $\alpha\gamma$ or $\alpha\delta$ dimers and conformational maturation of both the ACh binding site and the MIR. These results suggest that $\alpha\gamma$ and $\alpha\delta$ dimers may be normal assembly intermediates. β subunits did not assemble efficiently when coexpressed with α and γ or with α and δ subunits, but when α, γ, and δ subunits were coexpressed they efficiently assembled into unique complexes, and when all four types of subunits were coexpressed they efficiently assembled into mature AChRs. These results suggest that $\alpha\gamma$ and $\alpha\delta$ dimers can efficiently associate, and that assembly of these complexes with β subunits may be the rate-limiting step in AChR subunit assembly.

Materials and Methods

mAbs Used

mAb 35 to the MIR was initially described by Tzartos et al. (1981). Its epitope includes α68 and α71 (Saedi et al., 1990). mAb 210 to the MIR binds to an epitope within α68–76 (Das and Lindstrom, 1989; Saedi et al., 1990). Both mAb 35 and mAb 210 were made against native AChRs. mAb 142 was made against denatured AChR, and it was initially described by Tzartos et al. (1986). It binds to the cytoplasmic surface of *Torpedo* AChR α subunits at α360–366 (Ratnam et al., 1986*b*,*c*; Das and Lindstrom, 1990). mAb 111 was also made against denatured AChR (Tzartos et al., 1986). It binds to the cytoplasmic surface of β subunits within β360–410 (Ratnam et al., 1986*c*). mAbs were labeled with ^{125}I to specific activities of 1–10 $\times$ 10^{18} cpm/mol, as described in Lindstrom et al. (1981).

In Vitro Synthesis of AChR cRNAs

Torpedo AChR subunit cDNAs cloned under the control of SP6 promoter (Claudio, 1987) were a generous gift from Dr. Toni Claudio (Yale University). Plasmids were linearized by digestion with XbaI and used as templates in an in vitro transcription system using the SP6 RNA polymerase (Krieg and Melton, 1984). Samples were then treated with RNase-free DNase (20 μg/ml), extracted once with phenol/chloroform (1:1), and extracted twice with chloroform. The subunit-specific cRNAs were recovered by ethanol precipitation and dissolved in water before oocyte injection.

Expression of AChR Subunits in Xenopus Oocytes

Female *Xenopus laevis* were purchased from NASCO (Fort Atkins, WI), and the oocytes were prepared for microinjection as described by Colman (1984). Oocytes were injected with ~1.5 ng of each subunit-specific cRNA in various combinations and incubated at 19°C for 2–3 d to allow for the expression of AChR subunits. Oocyte extracts were prepared by homogenizing the oocytes in 50 mM Tris, 100 mM NaCl, 100 mM KF, 5 mM EDTA, 5 mM EGTA, 1.5% Triton X-100, 0.05% SDS, 5 mM 4-aminobenzamidine dihydrochloride, 10 μg/ml soybean trypsin inhibitor, 40 U/ml Trasylol, and 0.5 mM PMSF, pH 7.5. After incubating the homogenate at 4°C for 30 min, insoluble debris was removed by centrifugation in a microfuge at 4°C for 30 min. For sucrose gradient analysis, 100 μl of the oocyte extract (from 10 oocytes) was sedimented on a 5 ml 5–20% sucrose gradient at 65,000

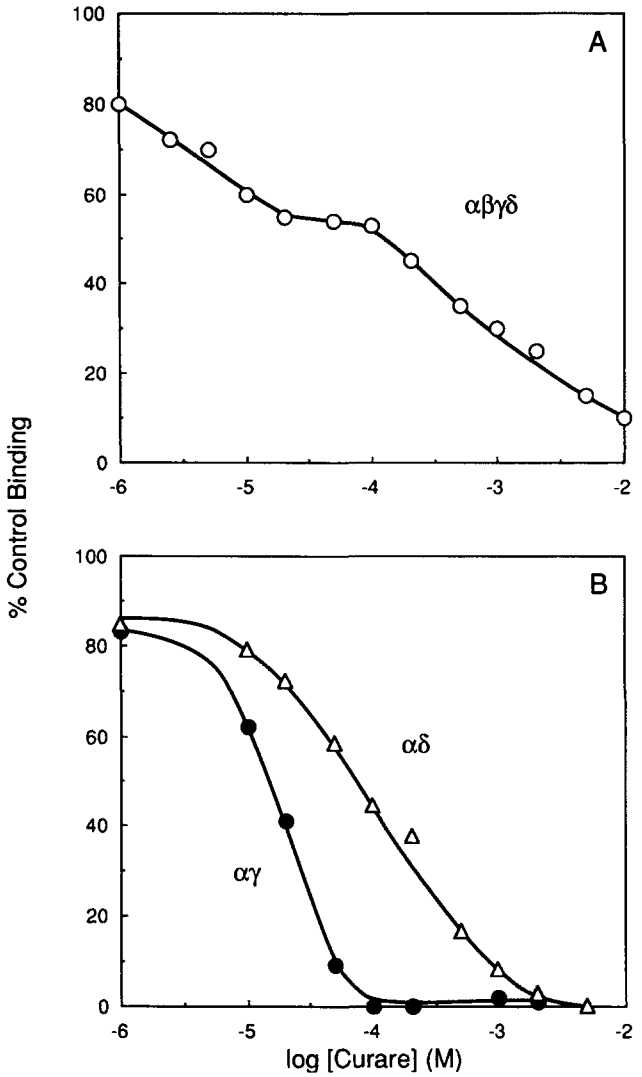


Figure 1. Analysis of curare inhibition of αBgt binding. Oocyte extracts expressing all four AChR subunits (*A*) or αγ (●) or αδ (△) subunit combinations (*B*) were incubated at 4°C in microtiter wells precoated with mAb 210, with different concentrations of curare and 2 nM ^{125}I-αBgt in a total of 100 μl. After an overnight incubation the bound concentration of αBgt was determined by washing each well four times and measuring the radioactivity by γ counting. Background equal to the binding of ^{125}I-αBgt remaining in the presence of saturating concentrations of curare was subtracted from each point. All data represent the average of three experiments.

rpm in a VTi 65.2 rotor (Beckman Instruments, Fullerton, CA) for 70 min at 4°C, and fractions (140 μl) were collected on microtiter plates coated with mAb 210. After an overnight incubation at 4°C with 2 nM ^{125}I-αBgt or 2 nM ^{125}I-mAb the microwells were washed four times with 200 μl of phosphate-buffered saline containing 0.5% Triton X-100, and radioactivity was measured by γ counting.

For examining the effect of curare on the inhibition of αBgt binding, 50 μl of oocyte extract (from approximately one oocyte) expressing various combinations of AChR subunits were added to microtiter wells coated with mAb 210. Samples were then incubated overnight at 4°C with various concentrations of curare and 2 nM ^{125}I-αBgt in a total volume of 100 μl. The amount of αBgt bound was then determined by washing each microwell four times with PBS containing 0.5% Triton X-100 and measuring the radioactivity by γ counting.

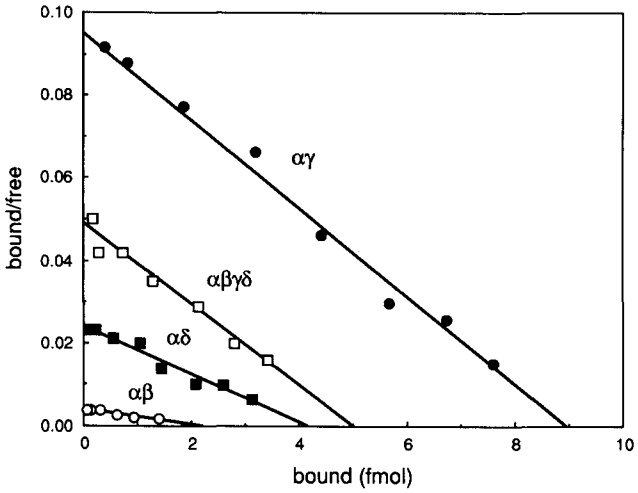
For Scatchard analysis of mAb 35 binding, 50 μl of oocyte extract (from approximately one oocyte) expressing the α subunit alone or in various combinations with other AChR subunits were added to microtiter wells precoated with mAb 142. Samples were then incubated overnight at 4°C with various concentrations (0.1-20 nM) of ^{125}I-mAb 35. The bound concentration of mAb 35 was measured by washing each microwell four times with phosphate-buffered saline-Triton and measuring the radioactivity by γ counting. Scatchard analysis was then performed using the EBDA program (Biosoft, Cambridge, UK).

In all experiments, the mAb coating of microtiter plates was accomplished by applying 50 μl of 10 mM sodium carbonate buffer, pH 8.8, containing 40 μg/ml mAb 142 or mAb 210 to each well, incubating the plates at 4°C overnight, and quenching with 200 μl of PBS containing 0.05% Tween-20 and 2% BSA for 2 h at room temperature.

Results

Conformation Change of the α Subunit at the Ligand Binding Site and the MIR

The two ACh binding sites of AChRs from *Torpedo* electric organ (Neubig and Cohen, 1979) and mouse BC3H-1 cells (Sine and Taylor, 1981) differ in affinity for curare. This difference is not detected in AChRs from *Electrophorus* electric organ (Prinz and Maelicke, 1983) or human TE671 cells



Subunit Combinations	Kd (nM)
αβγδ	1.0
αγ	0.9
αδ	1.7
αβ	5.5
α	>260

Figure 2. Affinity of mAb 35 for *Torpedo* AChR α subunits expressed in combination with other subunits in *Xenopus* oocytes. *Torpedo* AChR α subunits were expressed alone or in combination with the β, γ, or δ subunits of *Torpedo* AChR in *Xenopus* oocytes. Oocyte extracts were then added to microtiter plates precoated with mAb 142 and incubated overnight with various concentrations of ^{125}I-mAb 35. The bound concentration of mAb 35 was determined by washing each well four times and measuring the radioactivity by γ counting. Scatchard analysis was performed to determine the K_d values for each sample. Nonspecific binding was determined by performing the same analysis on extracts of noninjected oocytes and was found to be <10% of specific binding. All data represent the average of three experiments.

(Sine, 1988). Blount and Merlie (1989), by expressing pairs of mouse AChR subunits in fibroblasts, provided evidence that the molecular basis of the nonequivalence was the association of α subunits with γ subunits to produce the high-affinity site and the association of α subunits with δ subunits to produce the low-affinity site. Pedersen and Cohen (1990), by photoaffinity labeling *Torpedo* AChR with curare, provided evidence that amino acids of both α and γ subunits contribute to the site with high affinity for curare, while α and δ subunits contribute amino acids to the low-affinity site. Using the *Xenopus* oocyte system we tested, and confirmed, the idea that in *Torpedo* AChRs, association of α and γ subunits produces sites with higher affinity for curare, while association of α and δ subunits produces sites with lower affinity for curare. Native AChRs in extracts of oocytes expressing all four AChR subunits showed a biphasic curve indicative of two nonidentical binding sites for curare (Fig. 1 *A*), and extracts of oocytes expressing α and γ subunits had higher affinity for curare than did extracts of the oocytes expressing α and δ subunits (Fig. 1 *B*). The concentrations of curare giving half-maximal inhibition (IC_{50}) of αBgt binding for $\alpha\gamma$- and $\alpha\delta$-expressing oocytes were ~20 μM and 200 μM, respectively. Blount and Merlie (1989) also showed an ~10-fold difference in affinity for curare between mouse AChR $\alpha\gamma$ and $\alpha\delta$ subunit combinations expressed in quail fibroblasts. The IC_{50}s of αBgt binding inhibition observed for $\alpha\gamma$ and $\alpha\delta$ complexes do not absolutely equal the IC_{50}s observed for the low- and high-affinity binding sites for curare observed in oocytes injected with all four AChR subunits. The reason for this discrepancy is unclear, but it may be due to conformation changes induced by the assembly of all five subunits to form native AChRs. The important point is that specific association of α with γ subunits and α and δ subunits affects subtle properties of the ACh binding sites in ways qualitatively consistent with the properties of the native AChR.

To examine whether, upon association with other AChR subunits, the α subunit undergoes a conformation change at the MIR, the affinity for binding of mAb 35 in extracts of oocytes expressing the α subunit alone or in pair-wise combinations with the β, γ, or δ subunits was measured by Scatchard analysis (Fig. 2). mAb 35 is directed against the MIR and is highly conformation dependent, as it does not bind to synthetic peptides (Tzartos et al., 1988; Das and Lindstrom, 1989), to denatured *Torpedo* AChRs (Tzartos et al., 1981), or to α subunits as initially synthesized in BC3H-1 cells (Merlie and Lindstrom, 1983). Results showed that when expressed alone, the *Torpedo* AChR α subunit did not exhibit significant affinity for mAb 35. This is probably due to the unique instability of unassembled *Torpedo* α subunits (Paulson and Claudio, 1990) and contrasts with the properties of unassembled α subunits of AChRs from mice (Blount and Merlie, 1988) or humans (Conroy et al., 1990). Coexpression of α and β subunits yielded small amounts of low affinity binding of mAb 35. This may be due to association of some α subunits with β subunits, preventing the complete denaturation of the α subunits. The instability of unassembled *Torpedo* α subunits makes the observation of small amounts of low-affinity binding of mAb 35 difficult to interpret. Even in the case of mouse AChR subunits, Blount and Merlie (1989) have shown that α and β subunits do not associate efficiently: <5% of the α assembly intermediate

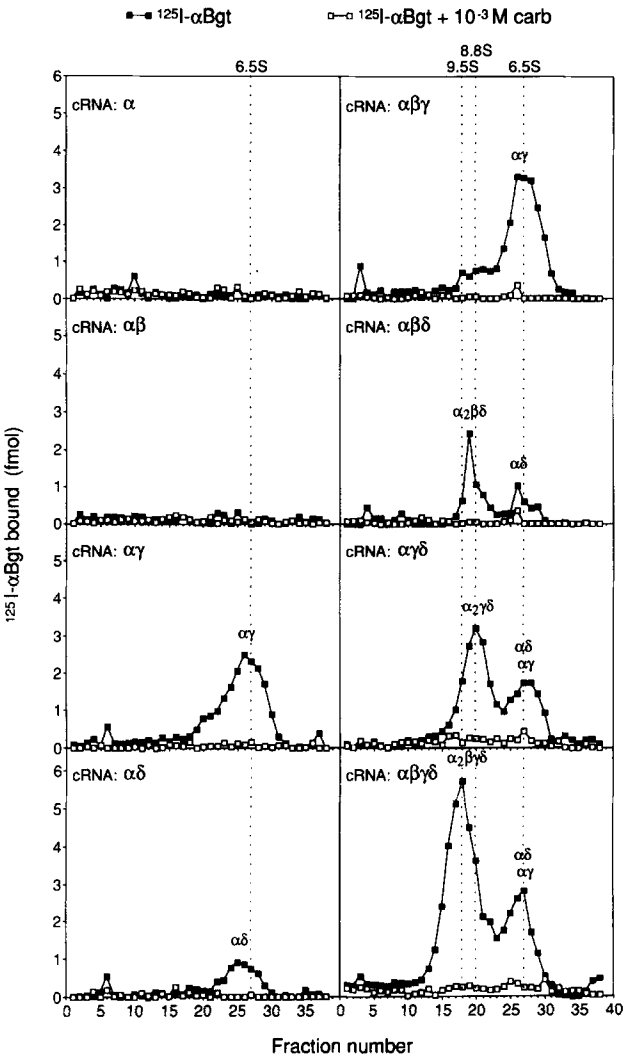


Figure 3. Cholinergic ligand binding by *Torpedo* AChR α subunit expressed in various combinations with other AChR subunits in *Xenopus* oocytes. Oocytes were injected with ~1.5 ng of cRNAs of each *Torpedo* AChR as indicated. 2 d later, Triton X-100 extracts of pools of 10 oocytes were sedimented on 5–20% sucrose gradients. Fractions were collected from the bottom onto microtiter wells coated with mAb 210 and analyzed for ^{125}I-αBgt binding activity in the absence (■) or presence (□) of 1 mM carbamylcholine. Fractions are numbered from the bottom of gradients. Each figure is the average of duplicate gradients, and each experiment was repeated three times with similar results. The S values were calculated using affinity-purified *Torpedo* AChR monomers (9.5S, fraction 18) and dimers (11.5S, fraction 12) as standards run on separate gradients at the same time. The rationale for assigning subunit stoichiometries is explained in the text.

could be immunoprecipitated by a β-specific mAb from fibroblasts expressing both α and β subunits. The dissociation constants (K_d) for the binding of mAb 35 in extracts of oocytes expressing both α and γ or α and δ subunits (K_d = 0.9 and 1.7 nM, respectively) were similar to the K_d for the binding of mAb 35 to native AChRs in extracts of oocytes expressing all four AChR subunits (K_d = 1.0 nM). Due to the instability of unassembled *Torpedo* AChR α subunits, it is unclear whether association with γ and δ subunits simply preserves the conformation of the α assembly intermediate

or enhances it. Since the affinity of unassembled human α subunits for mAb 35 is less than that of native AChR by ~15-fold (Conroy et al., 1990), it is likely that association with γ and δ causes maturation of the MIR from an intermediate to a virtually native conformation. This would closely resemble the effects of association of α with γ and δ subunits on the maturation of the ACh binding site.

Sucrose Gradient Analyses

To identify the complexes that are efficiently formed between AChR subunits, *Xenopus* oocytes were injected with cRNAs of the α subunit alone or in various combinations with other AChR subunits. 2–3 d later, oocyte extracts were prepared and the presence of various AChR subunits verified by western blot analysis using subunit-specific mAbs as probes (data not shown). Oocyte extracts were then sedimented on sucrose gradients and the gradients fractionated into 38 fractions onto microtiter plates coated with mAb 210. mAb 210 binds to the MIR on the extracellular surface of the α subunit at α67–76 (Das and Lindstrom, 1989). Affinity-purified *Torpedo* AChR monomers and dimers were sedimented on identical gradients as internal controls. To monitor the cholinergic ligand binding by various species that are formed, fractions were tested for ^{125}I-αBgt binding in the presence or absence of 1 mM carbamylcholine (Fig. 3). The α subunit expressed alone or in combination with the β subunit did not result in any species with significant affinity for αBgt. However, α subunit expressed in combination with γ or δ subunits resulted in the formation of a 6.5S complex with substantial affinity for both αBgt and carbamylcholine. Coexpression of α, β, and γ subunits resulted in the formation of a major αBgt-binding complex the same size as the αγ complex (6.5S) plus a heavier, but not distinct, minor peak which also bound αBgt and carbamylcholine. Coexpression of α, γ, and δ, or α, β, and δ subunits resulted in the formation of two distinct complexes which bound both αBgt and carbamylcholine. One complex was the size (6.5S) of αγ or αδ complexes, and another complex was ~8.8S (peak at fraction 20). Coexpression of all four AChR subunits resulted in the formation of two αBgt-binding complexes which also bound carbamylcholine: one complex at 6.5S (presumably αγ and αδ assembly intermediates) and another complex at 9.5S (native AChR monomers). Subjected to similar analyses, monomers of human AChR α subunit and native *Torpedo* AChR molecules sedimented at fraction 30 (5.0S) and fraction 18 (9.5S), respectively (Conroy et al., 1990). This indicated that AChR α monomers were displaced by 12 fractions to yield native *Torpedo* AChRs (a pentamer), suggesting that the association of each AChR subunit displaced the complex by approximately three fractions. This suggests that the peak at fraction 27 (6.5S) is composed of two AChR subunits, hence αγ or αδ pairs, and that the peak at fraction 20 (8.8S) is composed of four AChR subunits, e.g. $\alpha_2\gamma\delta$. We do not actually know the precise stoichiometry of any of the 8.8S peaks on Fig. 3, but the peak to which we attach the most significance is identified as $\alpha_2\gamma\delta$. This complex appears significant because it is formed efficiently in the presence of αγ and αδ subunits pairs which have acquired nearly native conformations of their ACh binding sites and MIRs, and it seems likely that association of two such subunit pairs would produce a complex of the observed size.

Because α subunits expressed alone in *Xenopus* oocytes

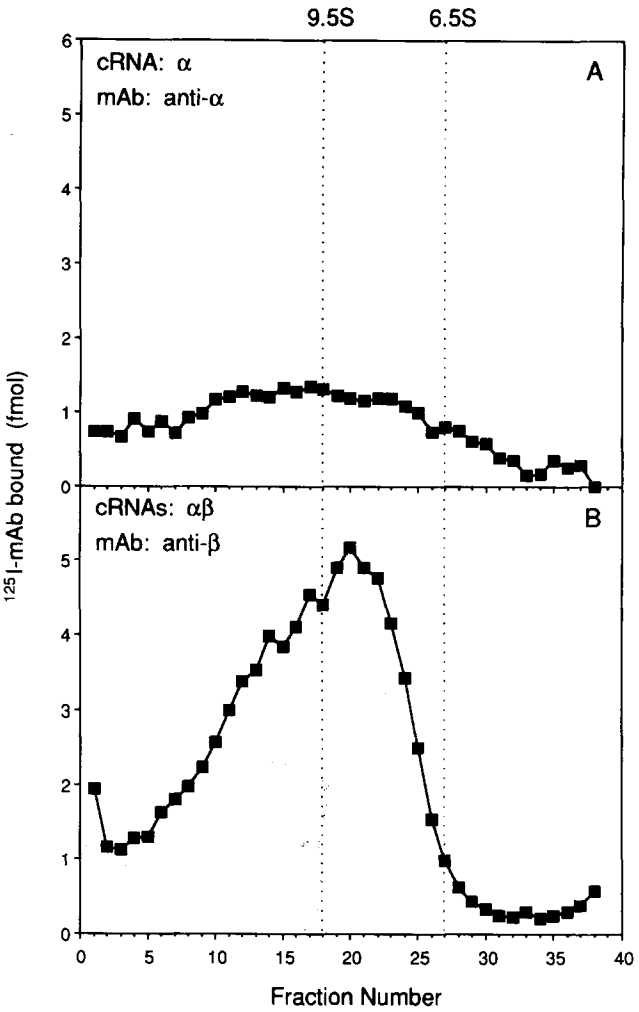


Figure 4. AChR α- and β-specific mAb binding by oocytes expressing AChR α alone or in combination with β subunits. Lysates of oocytes injected with AChR α alone (*A*) or AChR α and β subunits (*B*) were sedimented on 5–20% sucrose gradients. The gradients were then fractionated into microtiter plates coated with mAb 210 and analyzed for binding of ^{125}I-mAb 142 (α specific) or ^{125}I-mAb 111 (β specific) as indicated. Each figure is the average of duplicate gradients. Fractions are numbered from the bottom of gradients.

resulted in no detectable binding of ^{125}I-αBgt, the experiment was repeated using as a probe an mAb to α subunits which binds to both native and denatured α subunits. Binding of ^{125}I-mAb 142 (Fig. 4 *A*), as well as Western blots (data not shown), revealed that substantial numbers of α subunits were synthesized, and that these appeared to form complexes of a wide range of sizes that sedimented throughout the gradient. The observed complexes could reflect large homooligomers of the α subunit, as also observed by Anderson and Blobel (1983). Alternatively, the complexes could reflect nonspecific association of the α subunit with other proteins present in oocytes. In either case, they are clearly different from the 5S α subunit assembly intermediates described by Merlie and Lindstrom (1983) or Conroy et al. (1990), and are the denatured α subunits shown by Paulson and Claudio (1990).

Because coexpression of α and β subunits in *Xenopus* oocytes, like expression of α subunits alone, resulted in no de-

tectable binding of ^{125}I-αBgt, the experiment was repeated using as a probe ^{125}I-mAb 111 to β subunits to detect αβ-containing complexes anchored to microwells by the α-specific mAb 210. This analysis (Fig. 4 *B*), as with Western blots (data not shown), showed that substantial amounts of both α and β subunits were synthesized. These αβ complexes, like α subunits expressed alone, were found in a wide range of sizes which sedimented throughout the gradient. In this case also it is unclear whether these complexes are composed of α and β complexes only, or if they are composed of α and β subunits associated with various proteins present in *Xenopus* oocytes. It may be, as suggested for mouse α and β subunits (Blount and Merlie, 1989), that α and β subunits do not efficiently associate, that consequently both rapidly denature and aggregate with other proteins. The very short half life observed for *Torpedo* β subunits expressed in fibroblasts as compared with α, γ, or δ subunits (Claudio et al., 1989) is consistent with the idea that β subunits may be unstable and quickly denature if they cannot make appropriate associations.

To examine the association of β subunits with αBgt-binding complexes, we examined the sucrose gradient fractions of oocytes injected with α, β, and γ subunits or α, β, and δ subunits for binding by the β-specific mAb 111 to α-containing complexes anchored to microwells through mAb 210 (Fig. 5). When α, β, and γ subunits were coexpressed, very small amounts of β may have been associated with the 8.8S minor αBgt-binding peak, but most of the β formed amorphous aggregates with the α subunits that sedimented throughout the gradients (Fig. 5 *A*). When α, β, and δ subunits were coexpressed, a significant fraction of the β subunit associated with the 8.8S αBgt-binding peak, but much of the β subunit was found in amorphous aggregates with the α subunit that sedimented throughout the gradient (Fig. 5 *B*). When α, γ, and δ subunits were coexpressed, all of the α subunits were found to be efficiently assembled into the complexes thought to correspond to $\alpha\gamma$, $\alpha\delta$, or $\alpha_2\gamma\delta$, and no denatured α incapable of binding ^{125}I-αBgt was found in amorphous complexes (Fig. 5 *C*). This suggests that formation of complexes of α, γ, and δ subunits is very efficient and stabilizes α subunits, preventing the accumulation of denatured α aggregates.

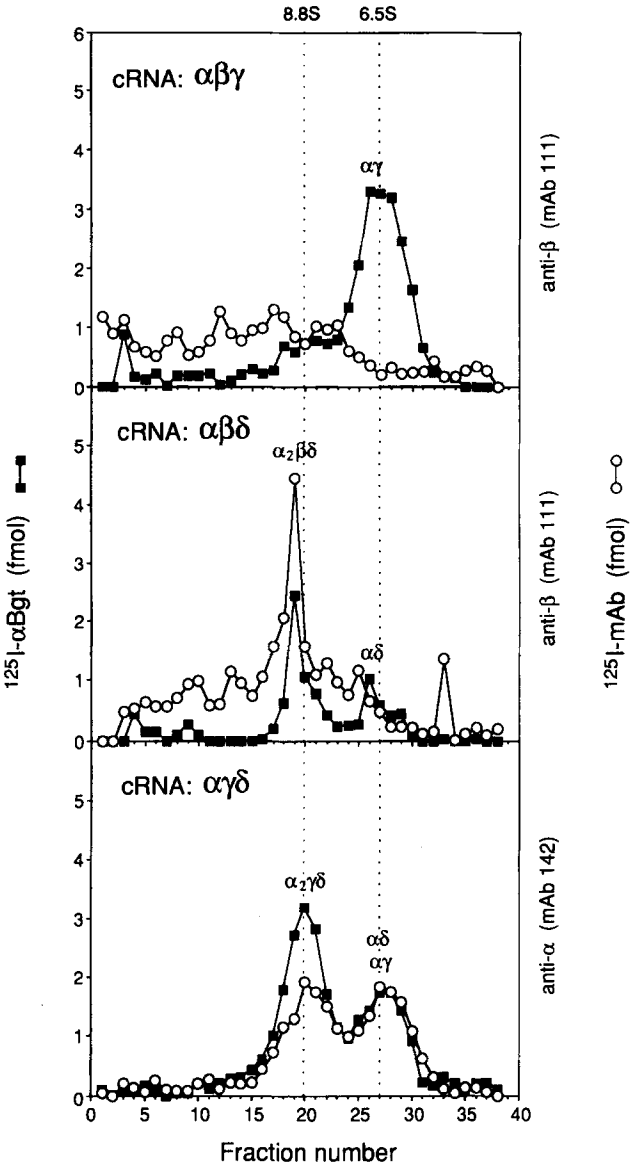


Figure 5. AChR α- and β-specific mAb binding by oocytes expressing AChR αβγ, αβδ, or αγδ subunit combinations. Extracts of oocytes injected with *Torpedo* AChR α in various combinations with other subunits as indicated were sedimented on 5–20% sucrose gradients. The gradients were then fractionated from the bottom onto microtiter plates precoated with mAb 210 and analyzed for binding of ^{125}I-αBgt (■) or for binding of ^{125}I-mAb 111 (β specific) or ^{125}I-mAb 142 (α specific) (○), as indicated. Fractions are numbered from the bottom of gradients. We have included the same toxin-binding curves presented in Fig. 3 to facilitate the comparison of the sizes and the positions of the peaks. Each figure is the average of duplicate gradients, and each experiment was repeated three times with similar results.

Discussion

Subunit assembly of multimeric proteins is a complex problem involving the association of individual subunits in a specific manner to form the mature protein with a unique subunit arrangement. All four types of AChR subunits exhibit extensive sequence homologies, suggesting that they have a common evolutionary origin and basically similar structures (Raftery et al., 1980; Noda et al., 1983). Thus, one might expect some degree of evolutionary conservation in the interfaces along which the subunits assemble. However, the subunits in native AChRs are strongly associated in a particular arrangement and cannot be dissociated easily except with SDS. It may be that all AChR subunits have some degree of low affinity for association with all other types as a result of their basically similar structures. However, when the proper pairs of subunits associate as they would in the native molecule, a conformation change could take place in one or both subunits which locks them together. According to this hypothesis, assembly-induced conformation changes would yield efficient formation of the productive complex and lead ultimately to assembly of an AChR with a unique, pseudosymmetric arrangement of subunits around the central channel, rather than to some labile arrangement in a stochastic mixture of orders.

α subunits synthesized in mouse BC3H-1 cells (Merlie and Lindstrom, 1983) or in human TE671 cells (Conroy et al.,

1990) efficiently mature after synthesis and before assembly to a 5.0S species with moderate affinity for both αBgt and mAbs to the MIR. However, *Torpedo* α subunits expressed alone in *Xenopus* oocytes, as shown here, form large complexes with a wide size distribution and lack the ability to bind αBgt. *Torpedo* α subunits expressed in oocytes may mature to assembly intermediates transiently, but if they are not stabilized by interaction with γ or δ subunits, they apparently denature and form the aggregates we observed. Paulson and Claudio (1990) also observed that α subunits expressed alone in fibroblasts do not bind αBgt or mAbs to the MIR.

When *Torpedo* α subunits were coexpressed with β subunits in oocytes, β subunits were found associated with α subunits (and perhaps other proteins) in complexes of a wide range of sizes. This association led to low amounts of α subunits with moderate affinity for mAbs to the MIR, but still undetectably low affinity for αBgt. This suggests that α subunits do not efficiently assembly with β subunits to form unique complexes that stabilize or cause conformational maturation of either subunit. These results are consistent with the observation that mouse α and β subunits expressed in fibroblasts do not efficiently associate or cause maturation of α subunits (Blount and Merlie, 1989). The observation that *Torpedo* β subunits expressed in fibroblasts turn over much more rapdily than do α, γ, or δ subunits (Claudio et al., 1989) suggests that β subunits expressed in the absence of subunits with which they can efficiently associate may be unstable and end up as denatured aggregates. In any case, our data do not suggest that α and β subunits assemble to form an important intermediate complex in the assembly of native AChRs.

When *Torpedo* α subunits were coexpressed with γ or δ subunits, they efficiently assemble into unique 1:1 complexes and no amorphous complexes incapable of binding αBgt were found. This suggests that α subunit assembly intermediates rapidly and irreversibly associate with γ and δ subunits and that this stabilizes the α subunits and prevents their denaturation. Association of α subunits with γ and δ subunits also causes a maturation in the conformation of the α subunits. The conformation of the ACh binding site matured to having the high afinity for carbamylcholine and curare which is characteristic of the native AChR. The conformation of the MIR also matured to that of the native AChR. These simultaneous conformational maturations at two widely separated parts of the extracellular domain of α subunits could reflect a global conformation change induced in α subunits allosterically through interaction with γ or δ subunits. The conformation could be slightly different in each case, as reflected in the different affinity of each subunit pair for curare, as is also characteristic of the two ACh binding sites in the native AChR (Blount and Merlie, 1989). A parallel global conformation change might take place in γ or δ subunits, but we currently lack the probes to detect it. These conformation changes might be the driving force for the assembly of the proper order of AChR subunits. Alternatively, there might be no conformation changes; instead, αγ and αδ subunit pairs might simply bind along certain interfaces with high affinity and their rigid polypeptide chains interdigitate to give the ACh binding site and the MIR their mature properties. Affinity labeling experiments have been interpreted to suggest this model in the case of the ACh binding sites (Pedersen and Cohen, 1990). In either case, formation of αγ and αδ subunit pairs may be normal steps in AChR assembly, which can proceed step-wise rather than requiring a concerted assembly of subunits.

When combinations of three subunits were expressed in *Xenopus* oocytes, we found significant differences in the efficiency with which specific complexes were formed. The most efficiently formed complex was found when the α, γ, and δ subunits were coexpressed, suggesting that this complex may represent a normal synthetic intermediate. This unique complex had a size consistent with a stoichiometry of $\alpha_2\gamma\delta$.

Coexpression of all 4 subunits led to efficient assembly of native AChR $\alpha_2\beta\gamma\delta$ monomers. It is interesting to note that if human α subunits (which efficiently mature to 5.0S assembly intermediates with moderate affinity for αBgt and mAbs to the MIR before assembly with γ or δ subunits) are substituted for *Torpedo* α subunits in the *Xenopus* oocyte expression system, mature AChRs are made in 5–10-fold greater amounts (Conroy et al., 1990). This suggests that in future studies of AChR subunit assembly in expression systems, it might be wise to avoid *Torpedo* α subunits. Some unassembled αγ and αδ pairs remain after coexpression of *Torpedo* α, β, γ, and δ subunits, which suggests that neither α, γ, nor δ subunits are rate limiting for assembly. It has been suggested from studies of *Torpedo* AChR subunits expressed in fibroblasts that β subunits have an exceptionally rapid rate of turnover and may be rate limiting in assembly (Claudio et al., 1989). This is consistent with our results, which suggest that, normally, α assembles efficiently with γ and δ subunits, and that these subunit pairs can efficiently assemble to form $\alpha_2\gamma\delta$ complexes which can very efficiently assemble into mature $\alpha_2\beta\gamma\delta$ AChRs, depending on the availability of β subunits. Further studies of this type will be required to sort out the details of AChR subunit assembly.

A simple model showing the primary synthetic intermediates in AChR subunit assembly which we and others have identified is shown in Fig. 6. The nascent α subunit has both an immature ACh-binding site and an immature MIR. The nascent α subunit has very low affinity for αBgt, negligible affinity for carbamylcholine and curare, and very low affinity for mAb 35 (Merlie and Lindstrom, 1983). Within 15–30 minutes after synthesis, some unassembled α subunits, as 5S monomers, partially mature to a conformation intermediate between that of the nascent polypeptide chain and the α subunit of the mature AChR. These α subunit assembly intermediates acquire moderate affinity for αBgt, but still lack affinity for carbamylcholine and curare, and they acquire moderate affinity for mAb 35 (Figs. 1–3; Merlie and Lindstrom, 1983; Smith et al., 1987; Blount and Merlie, 1988; Conroy et al., 1990). Assembly of α subunits into dimers with γ or δ subunits results in mature or nearly mature conformations of both the ACh binding site and the MIR. These 6.5S αγ and αδ subunit pairs acquire high affinity for αBgt, carbamylcholine, and curare, and the characteristic difference in affinity for curare of the two ACh binding sites is established (with αγ subunit pairs exhibiting higher affinity for curare); and αγ and αδ subunit pairs acquire equivalently high affinity for mAb 35 (Figs. 1–3; Blount and Merlie, 1989; Sumikawa and Miledi, 1989; Pederson and Cohen, 1990). α, γ, and δ subunits assemble efficiently into 8.8S complexes (Fig. 3). The size of these complexes suggests that their stoichiometry is $\alpha_2\gamma\delta$, but it is unclear whether the

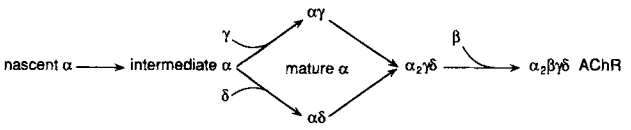


Figure 6. Primary synthetic intermediates in AChR subunit assembly.

subunits assemble from $\alpha\gamma$ and $\alpha\delta$ pairs into an $\alpha\gamma\delta\alpha$ (Kubalek et al., 1987) arrangement or into an $\alpha\gamma\alpha\delta$ arrangement (Blount and Merlie, 1989; Pederson and Cohen, 1990). In any case, several lines of evidence suggest that addition of β subunits is the final, rate-limiting, step required to complete assembly of AChR subunits around the central cation channel in native $\alpha_2\beta\gamma\delta$ stoichiometry: (*a*) β subunits do not assemble very efficiently with α subunits, do not efficiently stabilize α subunits, or cause their conformational maturation (Figs. 1–5; Blount and Merlie, 1989); (*b*) β subunits exhibit shorter half lives than the other subunits, suggesting that they may have a special role in assembly (Claudio et al., 1989); and (*c*) in addition to native AChR, TE671 cells express large amounts of unassembled, partially mature α subunits and detectable levels of $\alpha\gamma$ and $\alpha\delta$ subunit combinations, but no detectable small complexes of mature α with β subunits (Conroy et al., 1990). The actual process of AChR subunit assembly may be more complex than depicted in this simple model. Additional subunit complexes may contribute to the final assembly; for example, Figs. 3 and 5 show significant amounts of $\alpha_2\beta\delta$ complexes. Molecular chaperonins might play a role by loosely associating with subunit interfaces before their assembly into proper high-affinity complexes. Glycosylation clearly plays important roles in maturation, assembly, and transport (Merlie et al., 1982; Sumikawa and Miledi, 1989). Formation of disulfide binds (Conroy et al., 1990), lipid acylation (Olson et al., 1983), phosphorylation (Miles and Huganir, 1988), and other covalent modifications also probably play important roles yet to be difined in the maturation, assembly, transport, and turnover of AChRs.

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