THE MOLECULAR NEUROBIOLOGY OF THE ACETYLCHOLINE RECEPTOR

Michael P. McCarthy, Julie P. Earnest, Ellen F. Young, Seunghyon Choe, and Robert M. Stroud

Department of Biochemistry and Biophysics, University of California, San Francisco, California 94143

Introduction

The acetylcholine receptor (AChR) is the most thoroughly characterized component of the neuromuscular transduction process. Earlier reviews that summarize the structural and biochemical features of the AChR include Popot & Changeux (1984), Stroud (1983), Conti-Tronconi & Raftery (1982), and Karlin (1980). This receptor translates the binding of the neurotransmitter, acetylcholine (ACh), into a rapid increase and subsequent decrease in the permeability of the endplate membrane to the passage of cations. Inward flux of ions through the channel is passive, driven by electrochemical gradients across the receptor-containing membrane. The physiological effect is to temporarily depolarize the endplate, a response that is translated into muscular contraction in the case of a neuromuscular junction, or potentiation of electric tissue in the stacked asymmetric cells of electric organs in Torpedo (a marine elasmobranch) or Electrophorus (a freshwater teleost). The availability of acetylcholine receptors from electric tissue was a fundamental key to molecular characterization. The subunit stoichiometry of the four identified polypeptides has been unequivocally established as \( \alpha_2\beta_\gamma\delta \), and the funnel shape of the molecule has been well characterized with respect to position of the ion channel. Distribution of protein relative to the phospholipid bilayer and some aspects of the subunit arrangements in a quasipentameric structure around the ion channel have also been established. The genes for the four subunits that constitute the
minimal acetylcholine receptor from *T. californica* have been cloned and the amino acid sequences have been deduced. The amino acid sequence of the α subunits from *Torpedo*, bovine, and human sources shows the receptors to be close evolutionary homologues of one another. The α₂βγδ chains within any one species are more distantly related to each other than the α chain is between human and *Torpedo*, suggesting that the divergence of the four homologous chain types occurred early in the evolution of the synapse.

The neuromuscular nicotinic acetylcholine receptors are of interest as the target for autoimmune antibodies in myasthenia gravis, as the target for muscle relaxants used in surgical procedures, such as succinylcholine, and for studies related to development of the neural system. We here summarize what is known about the function of components in the system and the molecular characterization of the acetylcholine receptor, and the meaning of the structure for understanding the AChR at the molecular level.

**Organization of the Postsynaptic Membrane**

The postsynaptic membrane of the neuromuscular junction is predominantly characterized by its intricately folded structure and the high local concentration of AChR (Salpeter 1983). The high density of receptors is established in the postembryonic stage. Upon functional contact by a neuron at the nerve-muscle junction, previously diffuse AChRs in the postsynaptic membrane cluster at the junction, forming a tightly packed matrix of receptors (Ziskind-Conhaim et al 1984). Receptor molecules are localized at the crest of folds or invaginations of about 0.3–1.0 μm in depth, which occur across the face of the membrane. AChR density at the crest is about 10,000 AChR molecules per μm², or about 1 for every 100 × 100 angstrom area, in *T. californica* (Conti-Tronconi & Raftery 1982). An electron micrograph of a *T. californica* junction, demonstrating the relative orientation of the pre- and postsynaptic membranes and the basal lamina and the distribution of AChR molecules, is shown in Figure 1. Heuser & Salpeter (1979) observed the supramolecular organization of AChR molecules in rapidly frozen, deep-etched specimens. The advantage of this technique is that the specimen is frozen within the rotational relaxation time of a single molecule, and the structures revealed may be more biologically relevant than those seen with slower fixation procedures. Receptors occurred in strings of dimers, which associated to form four stranded strings and higher levels of organization in *T. californica*. In higher vertebrates, autoradiography and cytochemistry have shown that the AChR is concentrated at the crest (20,000–30,000 per μm²; Conti-Tronconi & Raftery 1982), whereas acetylcholinesterase is more uniformly distributed down and throughout the fold (Hartzell et al 1976). This implies
that the invagination does not increase the receptive area, as was suggested earlier, but may function instead as a diffusion "sink" for excess ACh. extrasynaptic receptors are distributed at only 0.1–0.01 times the surface density of junctional receptors and are electrophysiologically distinct (Peper et al 1982).

In a mature synapse, the integral membrane protein subunits recognized as the minimal components of the AChR are intimately linked with other proteins. Although co-distribution of receptors and heparin sulfate proteoglycans in the basal lamina has been observed in cultured skeletal muscle cells (Bayne et al 1984), the predominant interactions appear to involve cytoplasmic membrane proteins, generally referred to as the 43 kD or γ proteins. The distribution of these proteins, determined with immunoelectronmicroscopy, was seen to coincide with monoclonal antibody binding to AChR (Sealock et al 1984). The 43 kD proteins co-purify with the AChR, as seen by polyacrylamide gel electrophoresis, but can be removed by treatment with base (≥ pH 10.5) (Neubig et al 1979), demonstrating that they are only peripherally associated with the membrane. As the cholinergic response can be reconstituted by purification of only the four polypeptides α, β, γ, and δ of the AChR, the function of the 43 kD proteins is not yet clear. There are apparently three types of 43 kD protein, one of which probably has phosphatase activity (Gordon et al 1983). The removal of these proteins alters the ultrastructure of the cytoskeleton (Cartaud et al 1981) and accelerates the rotational diffusion of the AChR (Rousselet et al 1982). In addition, a 43 kD protein can be chemically crosslinked to the β subunit of the AChR (Burden et al 1983). The above information is consistent with a
model wherein the 43 kD proteins act largely to maintain the AChR in a closely-packed array at the synaptic crest.

X-ray diffraction reveals that the postsynaptic membrane has a lipid bilayer thickness of $40 \pm 1$ Å between phosphatidyl head groups (Ross et al 1977). The funnel-shaped receptor extends approximately 55 Å above the bilayer surface and provides an insulating environment for ions that enter the central ion channel (Klymkowsky & Stroud 1979). The lipid environment of *T. californica* electrocytes shows a fairly typical composition, approximately equimolar in cholesterol and phospholipids. It may be significant that the AChR-rich membranes contain more phosphatidyl ethanolamine, phosphatidic acid, and cholesterol, while the AChR-poor membranes have more phosphatidyl serine, sphingomyelin, and lysophosphatidyl choline (Gonzalez-Ros et al 1982). This study also demonstrated that the *T. californica* electrocyte lipid acyl chains contain an unusually high percentage of docosahexaenoic acid (22:6), although no significant differences were detected in the various fractions. *T. marmorata* electrocyte lipids have also been shown to contain high amounts of 22:6 fatty acids (Popot et al 1978).

**Shape and Size of the AChR Molecule**

The overall three-dimensional structure of the receptor has been analyzed by a number of methods. A three-dimensional model of the AChR is seen in Figure 2. The AChR was shown to be elongated by hydrodynamic measurement of the Stoke's radius of receptor-detergent complexes (Meunier et al 1972), assuming a receptor mass of 250,000 daltons. In neutron scattering studies, where the detergent component of solubilized AChR can be largely factored out, the receptor molecule was found to be cylindrical, with a radius of gyration of 4.6 nm (Wise et al 1979). The long axis of the receptor is perpendicular to the plane of the membrane. Small angle X-ray diffraction studies of oriented membranes determined that the overall length of the AChR was 11 nm, extending 5.5 nm beyond the extracellular surface of the membrane and about 1.5 nm into the cytoplasm (Ross et al 1977). The protrusion of the AChR molecule beyond the membrane was also seen in lateral views of receptor vesicles in the electron microscope (Klymkowsky & Stroud 1979). When viewed from above, the extracellular surface of the AChR appears as a rosette 80–90 Å in diameter. Each rosette contains a stained central pit (Cartaud et al 1978, Heuser & Salpeter 1979) of about 25 Å in diameter. The best evidence for this as the location of the ion channel is that it can be filled with stain to a depth of about 114 Å (Kistler et al 1982). This channel has been shown to conduct ions of less than 6.5 Å diameter at rates comparable to the rate of sodium ions (Maeno et al 1977, Dwyer et al 1980), defining a minimum diameter for
the most constricted portion of the channel. In a series of experiments in which transport of different diameter mono- and divalent cations through the channel was compared to free diffusion rates in solution, it was seen that the channel provides an environment very similar to the bulk aqueous phase (Lewis & Stevens 1983), showing that the channel is water-filled in its open state.

Although little is known of the tertiary structure of the AChR, the overall secondary structure is fairly well characterized. The existence of long $\alpha$-helices oriented perpendicularly to the membrane was deduced from small angle X-ray diffraction studies that showed characteristic 5.1 Å meridional and 10 Å equatorial peaks (Ross et al 1977). Circular dichroism studies of solubilized AChR molecules from *T. nobiliana* suggested a secondary structure that was 34% $\alpha$-helix, 29% $\beta$-structure (including turns), and 37% random coil (Moore et al 1974), while comparable studies with *T. californica* indicated 20% $\alpha$-helix, 50% $\beta$-structure, and 30% random coil (Mielke et al 1984). Resonance Raman spectroscopy of AChR molecules reconstituted into artificial membrane vesicles showed that the receptor from *T. marmorata* was 25% $\alpha$-helix (plus 14% disordered $\alpha$-helical ends) and 34% $\beta$-sheet (Aslanian et al 1983). Given the high degree of sequence

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**Figure 2** Three-dimensional model of AChR from *Torpedo californica*. The subunit locations around the central well are tentative assignments.
homology among these species, the secondary structure of the folded receptor is expected to be essentially the same for each of them as well. The membrane-spanning regions of the receptor are almost certainly \( \alpha \)-helical, whereas most of the extra-membranous regions of the AChR are probably composed of \( \beta \)-sheet and random coil as shown by Fourier transform analysis of sequence amphipathicity (Finer-Moore & Stroud 1984).

The quaternary structure of the AChR is a quasi-five-fold pentamer composed of four subunit types with the stoichiometry \( \alpha_2 \beta \gamma \delta \). This arrangement is evolutionarily conserved from \textit{Torpedo} and \textit{Electrophorus} to mammalian AChR (Raftery et al 1983), although the molecular weights of the individual subunits vary slightly. The molecular weight of the \textit{T. californica} AChR, calculated from the molecular weights of the subunits determined by gel electrophoresis and the known subunit stoichiometry, is 248,000 daltons (Weill et al 1974, Reynolds & Karlin 1978). This is apparently an underestimate (which can occur with glycoproteins), as a molecular weight of 268,000 daltons is suggested from the amino acid compositions of the subunits determined by cDNA analysis (Popot & Changeux 1984). In addition, the mature receptor is glycosylated (Vandlen et al 1979, Anderson & Blobel 1981) and phosphorylated (Vandlen et al 1979, Huganir et al 1984), and contains fatty acids linked to the \( \alpha \) and \( \beta \) subunits (Olson et al 1984), suggesting a final molecular weight of at least 290,000 daltons, since the sugar residues are estimated to total 20,000 daltons (Popot & Changeux 1984). As each subunit was shown to be exposed on both sides of the membrane by protease susceptibility (Wennogle & Changeux 1980, Strader & Raftery 1980) and to be externally glycosylated (Anderson & Blobel 1981), and accessible to hydrophobic probes (Middlemas & Raftery 1983), it is now clearly established that they are quasisymmetrically positioned in the native receptor, rather as staves in a barrel, each occupying a \( \sim 72^\circ \) segment (Fairclough et al 1983). Although the subunits show 40–50\% sequence homology with one another, they cannot substitute for one another. In in vitro expression systems, a significant response to ACh was seen only when the cDNA for all four subunits was available for expression (Mishina et al 1984). The arrangement of the individual subunits in the receptor is not known. The order \( \alpha \gamma \beta \delta \) was suggested from the angles between \( \beta \) and \( \delta \) subunits in artificially-generated trimers (Wise et al 1981), while the \( \alpha \) subunit was visualized with avidin-binding to biotinylated toxin (Holtzman et al 1982). A different subunit arrangement of \( \alpha \beta \gamma \delta \) was suggested on the basis of the binding of monoclonal antibodies, raised against the various subunits (Kistler et al 1982), and the position of \( \alpha \)-toxin binding relative to dimer contacts (Zingsheim et al 1982a) as observed in the electron microscope. It
is widely accepted that the α subunits are separated by one other subunit.

A final state of association observed between native AChR molecules in Torpedo species is dimerization, maintained by a disulfide bond between δ subunits. The amino acid residues involved have not been explicitly identified, but are probably the penultimate cysteines located at the carboxy-terminal ends of each δ chain (Wennogle et al 1981). Dimers have been observed to form parallel (Kistler & Stroud 1981, Bon et al 1984) or anti-parallel (Fairclough et al 1983, Brisson & Unwin 1984) doublets when tightly packed in the membrane, but they may interact more flexibly when diluted in membrane patches (reviewed in Popot & Changeux 1984). Dimers are only found in the AChR from Torpedo and Narcine, and do not occur in Electrophorus or vertebrates. Their significance is unknown; a number of workers have shown monomers and dimers to be functionally identical (Anholt et al 1980, Boheim et al 1981, Fels et al 1982), while other workers have shown that they are not (Schindler et al 1984, Chang et al 1984).

Electron microscopic images of individual particles have been analyzed by image analysis and statistical correlation techniques that rely upon maximizing the overlap of reproducible structural features in the molecules. Summed and averaged views as seen in the electron microscope describe a receptor surface of varying density, with the ridges and depressions suggested to represent the contribution of individual subunits (Zingsheim et al 1982b, Bon et al 1984), and the binding of α-toxin to the surface of the receptor has been localized, presumably to the α subunit. Studies of two-dimensional receptor "crystal" lattices formed in the plane of the membrane (Ross et al 1977) or in tubular crystalline membranes (Kistler & Stroud 1981, Brisson & Unwin 1984) allow the image of the AChR to be enhanced by Fourier transformation and image filtration. The results of such studies look rather similar to those based on single receptors. In studies on the better ordered tubes from T. marmorata to a nominal resolution of 30 Å, the surface of the receptor appeared to be a regular pentagonal rosette, with an asymmetric distribution of mass around the periphery (Brisson & Unwin 1984). Similar results are seen in studies of thin three-dimensional AChR crystals analyzed in the electron microscope (M. P. McCarthy, A. K. Mitra, S. Choe, R. M. Stroud, manuscript in preparation), whereas earlier studies described a somewhat more horseshoe-shaped projection, probably reflecting the expected asymmetric mass distribution between the subunits. Although these studies map the surface of the receptor rather well, detailed analysis of the three-dimensional structure of the AChR must await the growth of large, well-ordered crystals and the completion of high resolution X-ray diffraction studies.
Electrophysiological Action of the AChR

Descriptions of the response of the AChR to ACh (or other agonists) follow two general forms: electrophysiological measurements, primarily of vertebrate neuromuscular junctions, or biochemical characterizations of purified receptor from the electric organs of certain fish. While we concentrate on biochemical approaches to defining AChR structure and action, the following is a summary of the relevant electrophysiological data.

A typical presynaptic action potential at a neuromuscular junction induces the release of $0.2-3.0 \times 10^6$ ACh molecules into the synaptic cleft. Following a 300 μsec lag, about $2.5 \times 10^5$ channels will transiently open in the postsynaptic membrane, generating an endplate current of around $-400$ nA (Peper et al 1982). Overall open times for channels vary [extra-junctional AChR molecules stay open longer (Neher & Sakmann 1976)] but average about 1 msec at a membrane holding potential of $-100$ mV in frog neuromuscular junction (Magleby & Stevens 1972). This duration is sufficient to allow the net conductance of 10,000 Na$^+$ ions through each channel. Opening of the channel is caused by the binding of two or more molecules of ACh per receptor, as channel opening varies roughly with the square of the agonist concentration (Adams 1975, Dionne et al 1978), with a Hill coefficient of $1.97 \pm 0.06$ (Neubig & Cohen 1980). Average open times are also influenced by temperature and membrane potential; hyperpolarization of the membrane lengthens average open times (Magleby & Stevens 1972). Channel conductance values, however, average about 25 pS regardless of conditions in a variety of vertebrate neuromuscular junctions (Adams 1981).

The advent of patchclamp techniques (pioneered by Neher & Sakmann 1976) allows a uniquely detailed view of receptor function in the form of single channel recordings. Mean open times of individual AChR may be separated into two general classes in frog muscle, long-term (average 10 msec) and short-term (average 0.15 msec) (Colquhoun & Sakmann 1981). The authors suggested that the brief open forms may correspond to mono-ligated AChR. In addition, short non-conducting gaps occur during bursts that are too brief to be caused by agonist dissociation and subsequent rebinding. This flickering behavior (or Nachschlag) varies among species. In frog muscle, at low agonist concentration, there is an average of 3 gaps/burst, with a mean gap duration of around 40–70 μsec (Colquhoun & Sakmann 1981), while in snake twitch muscle, gaps are rarer (0.3 gap/burst) but longer (average duration of 200 μsec) (Dionne & Leibowicz 1982). The number of gaps per burst and mean open times are largely unaffected by membrane potential, but gap duration is shortened with hyperpolarization (Leibowitz & Dionne 1984). These gaps have been
claimed to describe excursions of doubly-liganded receptor into transiently nonconductive forms and may represent isomerizations that preceded the dissociation of bound ACh (Land et al. 1984). Contrary to the findings of earlier noise-analysis studies (Colquhoun et al. 1975), conductance does not vary with different agonists (Gardner et al. 1984); this suggests a simple two-state, open-closed system. In this case, the energy barrier between the two forms may not be large, as spontaneous openings of mouse AChR channels were shown to occur at infrequent intervals (Jackson 1984). In addition, the binding energy from antagonists such as the curare analogue, tubocurarine, can induce channel opening (Trautman 1982). However, the occurrence of "sublevel" conductance states, typically at lower temperatures or hyperpolarizing conditions, in embryonic rat muscle (Hamill & Sakmann 1981) and chick myoballs (Aracava et al. 1984) as well as in the presence of curare (Trautman 1982), demonstrates that the AChR is capable of adopting several open states.

Prolonged exposure of endplates to greater than micromolar concentrations of ACh results in a decrease of conductivity (Katz & Thesleff 1957). This phenomenon is known as desensitization and it varies in extent and duration with different agonists. It is not caused by direct blockage of the ion channel by agonist (Sakmann et al. 1980), although channel blockage by agonists may occur (Sine & Steinbach 1984). Desensitization may involve agonist binding to sites different from those involved in agonist activation. In frog muscle, desensitization rates (at 20 μM ACh) have been estimated at about 2 sec⁻¹; recovery is slightly faster (Sakmann et al. 1980). Much slower rates also correlated with desensitization have been observed, suggesting that the phenomenon associated with desensitization may involve more than one state or process (Adams 1981). Many drugs and local anesthetics initially suggested to increase desensitization rates have now been classed as noncompetitive channel blockers on the basis of electrophysiological analysis (Peper et al. 1982). Recovery rates from drug-enhanced desensitization appear to be drug insensitive (Magazanik & Vyskocil 1973), implying that drug dissociation is followed by a slow isomerization back to the resting state.

Electrophysiological studies on Torpedo AChR have been limited. Single channel recordings of T. marmorata AChR reconstituted into planar lipid bilayers showed mean channel open times of about 3 msec, similar to those seen in vertebrate endplates, with about four-fold higher conductance. The latter may be due to the high experimental ionic strength (Boheim et al. 1981). Analogous to observations at the vertebrate neuromuscular junction (Colquhoun & Sakmann 1981), T. californica AChR reconstituted into planar lipid bilayers exhibited two classes of open states, averaging 2.8 msec or 0.8 msec (Labarca et al. 1984). Furthermore, a difference in conduc-
tance dependent upon the state of association of the AChR has recently been described (Schindler et al 1984); the conductance of the monomer (20 ± 2 pS) falls within the range typical of vertebrate neuromuscular junctions, whereas the single channel conductance of a dimer appears two-fold higher. The authors suggested that the increased conductance of the associated species (which is not dependent upon covalent interactions) may reflect cooperative behavior important under physiological conditions. However, as noted above, a number of other investigators have failed to detect functional differences between Torpedo monomer and dimer preparations (Anholt et al 1980, Boheim et al 1981, Fels et al 1982).

**Biochemistry of Agonist and Antagonist Binding**

The number and importance of different agonist binding sites on the AChR remains a matter of some controversy. A majority of studies indicate the presence of two, high-affinity ACh binding sites per AChR monomer in Torpedo localized on the α subunit (reviewed in Conti-Tronconi & Raftery 1982, Popot & Changeux 1984). These sites may not be initially equivalent. The affinity reagents 4-(N-maleimido)benzyltrimethyl ammonium iodide (MBTA) and bromoacetylcholine (BAC) were found to label only one α subunit per AChR in *T. californica* AChR (Weill et al 1974, Damle et al 1978), whereas p-(trimethylammonium) benzenediazonium fluorobate labeled both α sites (Weiland et al 1979). In *T. marmorata* AChR, only one bromoacetylcholine binding site was reactive per monomer at 4°C, while both were labeled at 23°C (Wolosin et al 1980). Two classes of α subunits that differ in the extent of glycosylation have been observed in *T. californica* AChR (Conti-Tronconi et al 1984). These differences may contribute to the nonequivalence of the high affinity agonist binding sites. Although these sites may not be identical in structure, both are involved in channel opening. Even when one site is blocked by MBTA, ion flux could be induced by agonist binding to the second high-affinity site (Delegeane & McNamee 1980). ACh binding to these sites was shown to be weakly cooperative under desensitizing conditions (Fels et al 1982); models involving initially nonequivalent sites characterized by concave Scatchard plots (Prinz & Maelicke 1983) and long-lived, variable affinity states (Chang et al 1984) have been proposed. The Hill coefficient for channel opening is $1.97 \pm 0.06$ (Neubig & Cohen 1980), suggesting that the binding of two (or more) agonists is required for channel opening. The functionally significant number of binding sites is more difficult to determine, as most assays that correlate extent of binding with relevant operations such as ion flux are too slow, and thus probably describe the activities of largely desensitized receptor. One difficulty has been the contradictory values obtained for equilibrium carbamylcholine binding to the resting ($K_d = 30 \mu M$) and
desensitized states of purified receptor ($K_d = 10–100$ nM) (Weiland et al. 1977, Quast et al. 1978) and those determined to induce half-maximal activity in electrophysiological studies of frog muscle (0.5–1 mM) (Dreyer et al. 1978, Dionne et al. 1978). Improved ion flux measurements of purified receptor, utilizing $^{22}\text{Na}^+$ (Neubig & Cohen 1980) and $^{86}\text{Rb}^+$ (Hess et al. 1979) flux or $\text{Tl}^+$ quenching (Moore & Raftery 1980) yielded dose response curves similar to those determined by electrophysiological analysis. Complex kinetic schemes have been postulated to account for the differences observed in equilibrium and kinetic measurements (Neubig & Cohen 1980, Hess et al. 1983), and the possibility that the high affinity binding sites identified by MBTA are involved with desensitization while low affinity sites located on other subunits are responsible for channel opening has been suggested (Dunn & Raftery 1982b, Dunn et al. 1983). These researchers observed changes in the fluorescence of a bound, extrinsic probe indicative of a conformational change; this occurred with the time scale of channel opening even when the high-affinity sites were blocked by BAC and the receptor was in the desensitized state. Additional regulatory binding sites for agonists such as suberyldicholine on *Electrophorus* AChR (Pasquale et al. 1983) and a voltage-dependent inhibitory ACh binding site in *Electrophorus* (Takeyasu et al. 1983) and *T. californica* (Shiono et al. 1984) have been described. The location of these sites is not identified, but direct channel blockage, which is consistent with some electrophysiological measurements (Sine & Steinbach 1984), was assumed not to occur on the basis of model considerations.

The relatively slow transformation from low to high affinity states of the AChR seen in biochemical studies is thought to parallel the desensitization process observed electrophysiologically. Desensitization rates determined by changes in intrinsic protein fluorescence differ for the agonists suberyldicholine, carbamylcholine, and ACh in *T. marmorata*, but the final fluorescence state in each case is the same, implying that the ultimate form is independent of the ligand (Barrantes 1978). Desensitization may proceed from monoliganded receptors, as seen in fluorescent studies of Torpedo and *Electrophorus* (Bonner et al. 1976) and by kinetic analysis of ion flux measurements (Dunn et al. 1980, Hess et al. 1983). Two-step desensitization kinetics characterized by millisecond and second rates have been observed in reconstituted *T. californica* vesicles (Walker et al. 1982), again in agreement with electrophysiological data (Adams 1981). Local anesthetics (Weiland et al. 1977) and antagonists (Quast et al. 1978) have been shown to accelerate agonist-induced rates of desensitization in *T. californica*, although Covarrubias et al. (1984) did not see an enhancement in desensitization induced by competitive antagonists. Suberyldicholine binds more tightly to desensitized receptor than ACh, while carbamylcholine binds
more weakly, a relationship that extends to the average open times sustained by these agonists (Spivak & Albuquerque 1982).

Competitive antagonists such as cobra toxin and α-bungarotoxin (αBgTx) bind noncooperatively to receptor in B3CH-1 cells, but with Hill coefficients less than one, implying nonequivalent sites (Sine & Taylor 1981). However, in T. californica, both sites appeared equivalent in membrane-bound and detergent-solubilized AChR (Ellena & McNamee 1980). Snake toxins were found to bind with high affinity (average $K_d$ less than 0.1 nM; Weber & Changeux 1974) and their binding affinity seemed to be unaffected by desensitization (Weiland & Taylor 1979). They act by competitively blocking agonist binding and do not appear to interact with the ion channel. Curare, once considered a simple antagonist, appears to function as a partial agonist (similar to decamethonium)—both competing for (and activating) the agonist binding site and blocking open channel conductance (Trautman 1982).

The observed effect of agonist binding is channel opening and desensitization. On the molecular level, these processes involve conformational transitions of the AChR. ACh binding released 4–6 Ca$^{2+}$ ions (Chang & Neumann 1976) or 6–12 terbium ions (Rubasen et al. 1978) from purified Torpedo AChR. Agonist binding quenched intrinsic receptor fluorescence, at rates that suggested desensitization (Barrantes 1978, Bonner et al. 1976, Kaneda et al. 1982). Conversely, the fluorescence of noncovalently bound ethidium (Schimerlik et al. 1979) or the covalently bound fluorophore 5-(iodoacetamide) salicylic acid (Dunn et al. 1980) was enhanced by agonist binding. The sensitivity of a hydrophobic fluorophore, bound covalently to the β and γ chains of T. californica, to quenching by nitromethane was decreased by desensitizing concentrations of carbamylcholine, whereas α-bungarotoxin had the opposite effect (Gonzales-Ros et al. 1983). These studies do not allow definite conclusions to be formed about the conformational states of the AChR, but are consistent with a simplistic model wherein the agonist-binding site becomes less exposed to solvent upon occupancy, while the rest of the receptor becomes more accessible except for the membrane-spanning regions. Fluorescent studies such as these do not allow the extent of conformational transitions (local or global) to be estimated, although agonist binding was shown to increase the β-structure contribution in AChR CD spectra (Mielke et al. 1984). Tritium–hydrogen exchange experiments showed that the overall solvent accessibility of the resting and desensitized AChR were identical, whereas α-bungarotoxin binding restricted accessibility (M. P. McCarthy and R. M. Stroud, unpublished data). α-Bungarotoxin also induced the uptake of 4–6 Ca$^{2+}$ upon binding to receptor (Chang & Neumann 1976), enhanced desensitization rates (Quast et al. 1978), and modified the behavior of a
number of agonists and noncompetitive blockers (Spivak & Albuquerque 1982). Whether these effects are due to a large-scale conformational change or to local changes involving blockage of an essential binding site is not known.

**Location of Binding Sites**

The location of agonist binding sites remains a topic of great interest. It has been known for several years that covalent modification of reduced $\alpha$ subunits by affinity reagents permanently blocks agonist (and some antagonist) binding sites (Damle & Karlin 1978, Damle et al 1978). Recently, the target cysteine residue(s) have been identified as Cys 208 and perhaps Cys 209 (Kao et al 1984), amino acids unique to the $\alpha$ subunit in terms of sequence homology (Noda et al 1983a). The requirement for prior reduction suggests the existence of a cystine disulfide near the agonist binding site in the native AChR. Because cyssteines 208 and 209 follow one another in the sequence, Karlin and co-workers feel that these residues are unlikely candidates, as bridging between adjacent cysteines has never been observed in proteins; however, disulfide bonds are seen in crystal forms of a dipeptide, cysteinylcysteine (Capasso et al 1977), and are not otherwise energetically forbidden (Mitra & Chandrasekaran 1984). Instead, Kao et al (1984) suggested possible disulfide bonds with Cys 130 and 144 of the $\alpha$ subunit.

A sense of this binding site arises from analysis of the effects of agonists in terms of their structure. The bulky, quartenary amine head group of the agonist probably binds to a sterically-restricted, anionic region on the receptor, while hydrogen bond formation between ACh and AChR lend additional binding energy (reviewed in Spivak & Albuquerque 1982). Covalent cholinergic ligands have also been found to bind to the other subunits in unreduced AChR (Hucho et al 1976, Witzemann & Raftery 1977), perhaps reflecting low-affinity binding sites.

By definition, the competitive antagonist binding site overlaps the agonist binding site. In the case of snake neurotoxins (M.W. 7000–8000), this binding “site” is predicted to cover $20 \times 30$ Å of the receptor surface (Low 1979, Kistler et al 1982, Stroud 1983, Fairclough et al 1983). Binding of these toxins to the surface has been indirectly visualized in electron micrographs by complexing the toxins with gold-labeled antitoxin antibodies (Klymkowsky & Stroud 1979) and avidin binding to biotinylated toxin (Holtzman et al 1982), in the dimension perpendicular to the membrane by X-ray diffraction (Fairclough et al 1985), and in projection.

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1 The numbering of amino acids in this review follows the consensus alignment of homologous residues of Fairclough et al (1983).
using difference image autocorrelation methods (Zingsheim et al. 1982a). Snake toxins bind to the periphery of the receptor surface, away from the central pit, and are probably at least 50–60 Å apart from one another in AChR, with two toxins bound, as was seen in fluorescence energy transfer studies (Johnson et al. 1984). As the snake toxins are known to competitively block agonist binding to the high affinity site on the α chain, this is presumably their binding site as well. However, covalent cross-linking studies have not produced a consistent picture. α-Bungarotoxin derivatives have been shown to label both the α and δ subunits in T. californica (Witzemann et al. 1979) but in situ studies to crosslink all four subunits (Nathanson & Hall 1980). Given the size of the snake toxins, binding sites that involve more than one subunit are probable. Interaction of spin-labeled neurotoxin with the AChR suggested that Lys-27 of the toxin was in the vicinity of a disulfide on the AChR (Tsetlin et al. 1982). Lys-27 has been shown to be about 16 Å from the high-affinity agonist binding site (Fairclough et al. 1983), but which δ subunit cysteines form the disulfide is not known. Detailed descriptions of agonist and antagonist binding to receptor will require X-ray crystallographic analysis of receptor-ligand co-crystals.

**Sulfhydryl Groups**

A disulfide bond has been identified that, upon reduction and subsequent covalent modification, modulates agonist-stimulated ion flux by altering the affinity of the agonist for its binding site (Damle & Karlin 1978, Walker et al. 1984). Disulfide reduction by dithiothreitol (DTT) alone was found by Ben-Haim et al. (1975) to affect ion channel conductance, and by Moore & Raftery (1979) to alter the transition from the low-affinity to high-affinity state. Sulphonation was shown by Steinacker (1979) to increase miniature end-plate potential frequency and amplitude in cutaneous pectoris muscle of Rana pipiens. On the basis of competition with ACh, Steinacker proposed that the sulphonation site was in the vicinity of the ACh binding site. Affinity-labeling experiments using MBTA (Karlin 1980) suggested that the distance between the anionic sites where the quaternary ammonium of acetylcholine binds and the sulfhydryl groups where MBTA attaches is about 1 nm. Walker et al. (1984) demonstrated that reduction with DTT and subsequent alklyation with N-ethylmaleimide (NEM) or the more hydrophobic N-benzylmaleimide (NBM) dramatically decreased the flux response of asolectin-reconstituted AChR. In addition, alklyation by NEM was competitive with alklyation by the affinity reagent BAC, suggesting that NEM and NBM react with the same sulfhydryls as the affinity reagents MBTA and BAC. The above experiments confirm the existence of one or more sulfhydryl groups near the agonist-binding site. The cysteine residues
that form disulfide bridges have for the most part not been identified. Two cysteines in the N-terminal hydrophilic domain of the receptor are conserved in all four subunits and in all species tested. These cysteines (α 130 and α 144) can easily be in close proximity (Noda et al 1983a), as revealed by the identified turns in the β-pleated sheet domain (Finer-Moore & Stroud 1984), supporting the contention that these two cysteines form a disulfide linkage, based on the ability of the affinity labels MBTA and BAC to label residues 208 and 209 only after DTT reduction (Kao et al 1984). If Cys 130 and Cys 144 form a disulfide in the α subunit, the requirement for prior reduction suggests that either Cys 208 and Cys 209 form a disulfide between themselves, or that DTT treatment reduces other disulfides that block the accessibility of Cys 208 and 209 to affinity reagents.

Mishina et al (1985) have recently shown that mutation of any of the above-mentioned cysteines (130, 144, 208, or 209 on α) to serines completely eliminates the responsiveness of the expressed receptor to ACh. A mutation in either Cys 130 or Cys 144 completely prevents αBgTx binding, while a mutation in Cys 208 or Cys 209 reduces αBgtx binding to 28–39% of the control. These results may indicate that disulfide bonds involving residues 130 and 144 are critical for maintaining the tertiary structure of the hydrophilic domain that participates in toxin-binding.

There is evidence for one or more reactive sulfhydryl groups in the nonreduced receptor. Moore & Raftery (1979) found that para-chlor-mercuribenzoate (PCMB) modified the receptor in either the low-affinity or high-affinity state and blocked interconversion between states. This effect was seen in the absence of DTT treatment. Huganir & Racker (1982) demonstrated that the ability of various maleimides to inhibit agonist-stimulated ion transport was basically proportional to the hydrophobicity of the maleimide. This phenomenon was examined in detail by Walker et al (1984), who found that 0.4 mM NBM blocked 50% of the carbamylcholine-stimulated 86Rb+ influx in Torpedo AChR reconstituted into asolectin. The concentrations of NEM and iodoacetamide required to block 50% of the flux response were about 60 and 400 mM, respectively (based on their Figure 10). Toxin competition assays performed on NBM-modified membranes established that the inhibitory effect of NBM modification was not correlated with impaired ligand binding. These results, along with those of Huganir & Racker (1982), implicate a hydrophobic site associated with a sulfhydryl group that is capable of modulating the function of the ion channel.

Not only is there evidence that sulfhydryls can modulate channel activity, but recent evidence has shown that a conformational change in the receptor
alters susceptibility of sulfhydryls to alkylation. Otero & Hamilton (1984) demonstrated that labeling of different subunits of AChR-rich membranes from *T. californica* with [³H]NEM changes upon addition of cholinergic ligands. The native receptor, with or without carbamylcholine, was labeled only on the β subunit, whereas reduced receptor was labeled on the α and β subunits. The addition of carbamylcholine, and to a lesser extent choline, αBgTx and curare to the reduced receptor resulted in a decreased labeling of the α subunit, with no change in the labeling of the β subunit. Gallamine, which has been shown to block agonist-induced ion flux both as a competitive antagonist and in an alternate voltage-dependent fashion, increased NEM labeling of the α subunit up to twice the value of the reduced control, suggesting that gallamine makes additional disulfides accessible to DTT reduction. The small decrease in α-labeling caused by αBgTx, relative to the large decrease in α-labeling caused by carbamylcholine, suggested to these researchers that steric hindrance of a reducible disulfide in the presence of cholinergic ligands is not responsible for the observed differences in NEM-labeling. Their observations are consistent with ligand-induced conformation changes altering accessibility of sulfhydryl groups.

**Noncompetitive Blockers**

Compounds that block or modulate the agonist-stimulated increase in cation permeability, but do not bind at the acetylcholine site (at pharmacologically relevant concentrations) include amine local anesthetics, histrionicotoxin (from the poison-dart frog of South America), the psychoactive tranquilizer phencyclidine, nonionic detergents, the antipsychotic chlorpromazine, and aliphatic alcohols (Spivak & Albuquerque 1982). These diverse compounds are not likely to have the same binding site or mechanism of action. There appear, however, to be several properties common to noncompetitive blockers.

Noncompetitive blockers stabilize the AChR in the desensitized state. The affinity of the AChR for some noncompetitive blockers is enhanced by binding of agonists and some antagonists; binding of [³H]phencyclidine to *Torpedo* AChR-rich membranes was 10³–10⁴-fold faster when carbamylcholine was added concurrently (Oswald et al 1984). Binding of local anesthetics to saturable site(s) on the AChR stabilized the receptor in a state of high affinity for agonist (Heidmann & Changeux 1979, Boyd & Cohen 1984). Agonists and local anesthetics (but not competitive antagonists) induced the transition of the AChR to the desensitized state (Covarrubias et al 1984). It is unclear whether the stabilization of the desensitized state is the primary pharmacological effect of noncompetitive blockers.

Noncompetitive blockers bind to multiple sites on AChR membranes. A
saturable, high-affinity, allosteric binding site for noncompetitive blockers has been proposed (Heidmann et al. 1983, Oswald et al. 1983, Haring & Kloog 1984). Two classes of binding sites for $[^3]H$phencyclidine on acetylcholine receptors from T. ocellata membranes have been identified: one high-affinity ($K_d = 6–9 \mu M$) site per receptor, and two low-affinity ($K_d = 85 \mu M$) sites per receptor (Haring & Kloog 1984). Binding of $[^3]H$phencyclidine to the low-affinity sites could be completely blocked by the presence of $[^{125}]\alpha$-bungarotoxin. The results were consistent with the low-affinity site being the agonist-binding site, and evidence was presented to suggest that at close to millimolar concentrations, noncompetitive blockers can bind to the acetylcholine site and promote desensitization. Heidmann et al. (1983) found that high-affinity binding of phencyclidine, meproadifen, and the detergent Triton X-100 could be competitively blocked by perhydrohistrionicotixin with a fixed stoichiometry of one site per receptor monomer, while binding of chlorpromazine and trimethisquin was less sensitive to perhydrohistrionicotixin, had lower affinity, and showed 10 to 30 sites per monomer. The number of low affinity sites was linearly dependent on the lipid-to-protein ratio in reconstituted membranes, suggesting that these low affinity sites are at the lipid-protein interface. The affinity of any noncompetitive blocker for the low-affinity site(s) is a function of the molecule's solubility in the lipid bilayer, or more importantly its solubility at the lipid-protein interface, and of the charge on the molecule. A third "nonsaturable" binding site in the bulk phase lipid was also proposed. The high lipid-solubility of several of the noncompetitive blockers suggests that even if there is one saturable high-affinity binding site per receptor monomer that is responsible for blocking agonist-stimulated ion flux, the lipid membrane provides the receptor with a locally high concentration of drug, which may have its own effects on receptor function through specific effects on the lipid-protein interface, or by affecting the local charge environment.

Recent experiments using inside-out cell patches and both tertiary and quaternary amine noncompetitive blockers demonstrated that a positive charge on the noncompetitive blocker is necessary for pharmacological effect at micromolar concentrations, and that the drug interacts with the AChR from the extracellular side of the membrane (Aracava et al. 1984, Aracava & Albuquerque 1984). That the positively charged form of a tertiary amine local anesthetic binds with high affinity to reconstituted AChR and blocks ion flux has been corroborated by Earnest et al. (1984) and Blickenstaff & Wang (1985).

Electrophysiological studies have shown that noncompetitive blockers do not affect single-channel conductance, but do decrease channel lifetime; the effect is generally voltage-dependent (Koblin & Lester 1979,
Albuquerque et al 1980, Lambert et al 1983), although voltage-independent blockage has been seen recently by Ribera et al (1985). The observation that the binding of some noncompetitive blockers to site(s) on the AChR is dependent upon the applied voltage has led to their further classification as "open channel blockers." Although some of the data can be explained most easily by direct occlusion of the channel by noncompetitive blockers, there is no direct evidence for binding site(s) for these compounds within the ion channel.

Attempts have been made to identify local anesthetic binding sites on the AChR using photoaffinity labels (Lester et al 1980, Oswald & Changeux 1981a,b, Heidemann & Changeux 1984, Muhn et al 1984) and alkylating derivatives of local anesthetics (Kaldany & Karlin 1983). Quinacrine mustard was shown by Kaldany & Karlin to block channel function at low concentrations (10 μM) without blocking agonist binding. Tritiated quinacrine mustard labeled the α and β chains of the receptor under these conditions. Kaldany & Karlin's results implicating the α and β chains as the sites of functionally significant local anesthetic binding differ from the results of Oswald & Changeux (1981a), which implicate the δ chain. In the latter the label was a radioactive photoaffinity derivative of the local anesthetic, trimethisoquin (5-azido[3H]trimethisoquin), and its incorporation into the α chain was inhibited by cholinergic agonists and antagonists. Binding to the δ subunit was competitive with nonlabeled trimethisoquin and with [3H]phencyclidine. [3H]chlorpromazine irradiated with UV light showed an agonist-dependent rapid covalent incorporation into all five subunits, suggesting a site within the ion channel that becomes accessible when the channel is open (Oswald & Changeux 1981b, Heidmann & Changeux 1984). Photolabeling experiments of amphiphilic compounds such as local anesthetics have the intrinsic problem that rapid exchanges among lipids, proteins, and drugs in the lipid phase make identification of a specific binding site difficult. Muhn et al (1984) have attempted to resolve the interaction of AChR with [3H]triphenylmethylphosphonium (a lipophilic cation thought to block the AChR ion channel) in millisecond-to-second time scales using a stopped-flow apparatus and a high-energy pulse laser. In the absence of cholinergic ligands, most of the label was incorporated into the α subunit. With cholinergic ligands, labeled anesthetic was incorporated into the δ and somewhat into the β subunits, although the α subunit still contained the majority of the label.

Discrepancies in labeling patterns could be due to differences in the time scale of interactions, or variability in the effects of the ligands on receptor function. (Functional effects of the label under investigation on agonist-stimulated ion flux were examined only by Kaldany & Karlin.) Moreover, it
is possible that there are distinct binding sites for different noncompetitive inhibitors. Although binding sites for noncompetitive blockers may be identified, further research is required before the pharmacologically relevant binding site(s) is identified or understood.

**Sequences of the Subunits**

The full sequences of all four subunits in the AChR have now been determined and have proved uniquely important in providing structural detail and insights into function. Sequencing began at the amino acid level. Devillers-Thiery et al (1979) sequenced the first 20 amino acids at the N-terminus of the α chain of AChR from *T. marmorata*. Raftery et al (1980) determined the first 56 amino acids of each subunit from *T. californica*, and by quantitative comparison proved the stoichiometry of the chains as α₂βγδ. There is 100% correspondence between the first 20 amino acids of the α chain from *T. marmorata* and *T. californica*. Raftery et al (1980) further showed that all four subunit types have 35–50% sequence homology with one another in this region. Using synthetic oligonucleotide primers for short stretches of amino acids, Ballivet et al (1982) with the γ chain and Sumikawa et al (1982) with the α chain reported cloning and sequencing of the entire precursors of these chains, including the N-terminal signal sequences deduced from cDNA clones. Subsequently, Numa and his colleagues obtained the sequences for *T. californica* α subunit (Noda et al 1982), and then by similar techniques the β and δ subunit precursors (Noda et al 1983b). Sequencing of the γ subunit by Claudio et al (1983) yielded a proposed topological model for the chain that contained four very hydrophobic stretches about 26 amino acids in length, long enough to span the lipid bilayer in α helical conformation. Cloning of the *T. marmorata* α chain cDNA by Giraudat et al (1982) led to a similar topological model, and to the proposal that the most polar of the four hydrophobic helices within each chain could contribute to formation of an ion channel between all five subunits (Devillers-Thiery et al 1983). The orientation of the large, amino terminal region involved in agonist binding, the four hydrophobic membrane-spanning regions (M1-M4), the amphipathic channel forming region (A1), and the cytoplasmic regions of a consensus subunit are depicted in Figure 3. The evolutionary relatedness of the different chains was dramatically displayed by Noda et al (1983a) following their complete sequencing of all four chains. After alignment they were shown to be 19% identical at equivalent sites and 54% homologous, in that three (or four) chains with identical residues have a conservative substitution in the divergent chain. Comparison of *Torpedo*, calf, and human subunit sequences led Numa and co-workers to propose that the α subunit has evolved more slowly than the other subunits, and that the basic α₂βγδ subunits
stoichiometry was established 550–690 million years ago (Kubo et al 1985).

Determination of these sequences revolutionized thinking about the AChR. Noda et al (1983c) showed a 97% homology between α chains of human and calf, and 80–81% homology between human and Torpedo α chains. The functional AChR is highly conserved throughout evolution, as the recent determination of the sequences of mouse δ chains (La Polla et al 1984) and chick γ and δ chains (Nef et al 1984) has confirmed.

Examination of the splice junctions in both human genomic (Noda et al 1983c) and in Torpedo genomic sequences (Noda et al 1983a) showed that the 9 exons in human α gene and 12 exons in Torpedo γ and δ in some cases precisely corresponded to predicted structural domains of the receptor subunits; in all cases there are introns between the predicted hydrophobic membrane spanning regions M1 and M2, 15 amino acids beyond M3, and 13 amino acids prior to M4. In chicken and Torpedo another exon lies between M2 and M3. Thus the pattern of functional domains and specifically the transmembrane sequences being encoded in separate exons is strongly indicated in this membrane protein.

Analysis of the amphipathic character of secondary structures demonstrated one sequence (A1) just before M4 that, if in α helical

![Figure 3 Secondary structure and topography of an acetylcholine receptor subunit. Hydrophobic, transmembrane α-helices are numbered M1–M4, and the amphipathic putative channel-forming α-helix is labeled A1. Possible glycosylation sites are marked with stars, and residues whose charge is conserved in all four subunits are depicted. Residue numbering follows the sequence alignment of homologous amino acids of Fairclough et al (1983).](image-url)
conformation, would present one highly charged surface and one very hydrophobic surface (Stroud 1983, Fairclough et al 1983, Finer-Moore & Stroud 1984, Guy 1984), which suggested that the five subunits could each contribute one highly charged surface to the conformation of a central, water-filled, ion-conducting channel across the membrane.

Mishina et al (1984) subsequently obtained expression of their cDNA clones for all four subunits of the Torpedo AChR. The procedure involved production of mRNA under an SV40 promoter in sensitive COS monkey cells. The mRNA was subsequently injected into Xenopus oocytes and expressed. In 35 site-specific mutagenesis experiments aimed at alterations in the \( \alpha \) chain of Torpedo AChR, Mishina et al (1985) showed that certain regions of the sequence are very important for different functions. Substitution of cysteine residues 130 or 144 for serine eliminated ACh-induced ion flux and reduced bungarotoxin binding to only 8%. The authors suggested that the extracellular \( \beta \)-sheet domain may be unstable without the putative S–S bond between these residues. Replacement of cysteine by serine at positions 208 or 209, shown by Kao et al (1984) to lie at the MBTA affinity-labeled site, destroyed ACh-induced ion flux sensitivity but only diminished \( \alpha \)-BgTx binding to 28–39% relative to native receptor, consistent with the large binding surface predicted for \( \alpha \)-BgTx binding (Low 1979, Kistler et al 1982). Substitution of 5–10 amino acids by 0–3 amino acids in M1, M2, or M3 resulted in no detectable ACh sensitivity and had a profound effect on bungarotoxin binding, possibly by affecting assembly of the AChR complex. Almost all substitutions in the region of the amphipathic helix (A1), which may contribute to ion channel formation, eliminated ACh sensitivity, while reducing toxin binding by 56–70%. The one exception was that deletion of residues 417–452, which comprise the entire amphipathic region, showed a slight (\( \sim 3\% \)) ion-fluxing capability in response to ACh. Mishina et al (1985) pointed out that the neighboring region, which may be dragged across the membrane during insertion, is also of amphipathic quality and could conceivably substitute for region A1. Evidently A1 is essential for ion flux, but is less crucial for inhibition of \( \alpha \)-BgTx binding by carbamylcholine. Small substitutions within either the carboxy terminal sequence after M4, or within most of the cytoplasmic domain between residues 324–434, did not substantially impair functional properties.

**Topography of the AChR in the Bilayer**

With the amino acid sequences of all four subunits from *T. californica* known, a significant amount of topographical information can be deduced by analyzing the results of biochemical, immunological, and proteolytic
studies. Each subunit contains a large amino terminal region ($M_r \sim 30,000$ daltons) that contains the sites for core glycosylation (Anderson & Blobel 1981, Anderson et al 1983). The most likely site for $N$-glycosylation is Asn 143, which is conserved in all four subunits (Noda et al 1983a). This hydrophilic domain is extracellular, as shown by protease susceptibility (Wennogle & Changeux 1980), and contains the high affinity agonist and antagonist bindings sites in the $\alpha$ subunit (Kao et al 1984). The amino terminal region corresponds to the portion of the receptor seen to extend 55 Å above the membrane on the synaptic side (Klymkowsky & Stroud 1979, Kistler et al 1982).

The first three of the four hydrophobic membrane-spanning regions (M1–M3) follow one another rather closely in the sequence, connected by short hydrophilic loops approximately 5 and 16 amino acids long, respectively (Claudio et al 1983, Devillers-Thiery et al 1983, Noda et al 1983a). The short loop between M1 and M2 is predicted to be cytoplasmic, and the longer loop connecting M2 and M3 should lie on the synaptic side of the membrane. The disposition of the long segment (approximately 155 amino acids in length) between M3 and M4 is largely cytoplasmic. The amphipathic helical segment A1 introduces a fifth membrane crossing, reduces the length of the large cytoplasmic segment to about 110 residues, introduces a short extracellular loop of about 20 amino acids between A1 and M4, and places the carboxy terminus of each subunit within the cytoplasm.

The initial portion of the link between M3 and M4 has been shown to be cytoplasmic by several groups. Wennogle et al (1981) generated a 16,000 dalton segment of the $\delta$ subunit, corresponding to the carboxy-terminal region, which included A1, M4, and some of the cytoplasmic linker, and showed that the site of cleavage was cytoplasmic by the disposition of phosphorylation sites. Huganir et al (1984) demonstrated the cytoplasmic location of the phosphorylation site, and have tentatively identified the specific phosphorylation sites as serines and tyrosines between residues 364–381 in all four subunits. Barkas et al (1984) generated antibodies to peptides corresponding to segments 153–184 and the carboxy-terminal segment 489–500 of the $\alpha$ chain, and showed that cleavage between the binding sites for these antibodies was enhanced by sonication, indicating that the cleavage site was cytoplasmic. More specifically, by using a colloidal-gold second antibody to visualize binding, antibodies raised against a peptide corresponding to residues 350–358 of the $\beta$ chain (45 residues from the end of M3 and 32 residues from the start of A1) were shown to bind to the cytoplasmic side of the postsynaptic membrane in disrupted $T.\ californica$ electroplaque (Young et al 1985).
The cytoplasmic location of the carboxy terminus of receptor subunits, which is consistent with the five-crossing model, has recently been demonstrated; Lindstrom and co-workers (Lindstrom et al 1984, Ratnam et al 1984) raised antibodies to peptides corresponding to the carboxy termini of all four subunits, and showed that detergent or lithium diiodosalicylate permeabilization of AChR vesicles was necessary for antibody binding. In vitro studies performed by Young et al (1985) using antibodies raised against the carboxy-terminal residues 501–516 of the δ subunit also showed that detergent solubilization of AChR vesicles exposed the carboxy terminus to antibody binding. In addition, the cytoplasmic location of the δ carboxy terminus was demonstrated in situ, in Torpedo electroplaque, by visualizing antibody binding in the electron microscope through use of colloidal-gold second antibodies. This proved that the carboxy terminus was cytoplasmic, and that detergent solubilization was not acting simply to expose an extracellular carboxy terminus that was buried in the protein. The cytoplasmic location of the δ carboxy terminus seems to require that the δ–δ disulfide bond in dimeric AChR must also be cytoplasmic.

**Nature of the Suggested Ion Channel**

The amphipathic sequence suggests that charged residues contributed by similar and homologous sequences from five subunits, present on one side of a 50 Å long α-helix within each subunit, could come together to form the lining of the ion channel. The five-fold symmetrical arrangement implies that similar residues will be brought together at similar heights relative to the bilayer, and that the channel lining would contain alternate layers of positively and negatively charged residues. While the net charge within this 40 Å transmembrane region would be neutral, the entrance would be predominantly negatively charged, and so provide specificity for cations, as anions would be repelled. The funnel-shaped structure of the molecule would also provide some insulation against the effects of membrane surface charge. Model-building experiments show that five α-helices brought together with five-fold symmetry and placed 11 Å apart as suggested by X-ray scattering (R. H. Fairclough and R. M. Stroud, unpublished results) would generate a central channel no less than 7.0 Å across. This diameter closely parallels the value of 6.5 Å estimated by electrophysiological measurements for conductivity of organic cations (Maeno et al 1977, Dwyer et al 1980). Such an arrangement of close-packed helices can accommodate passage of a hydrated sodium ion or an organic cation such as diethyldimethylammonium, simply by movement of side chains away from the center of the channel. Electrostatic energy calculations suggest
that the barriers to passage of monovalent or divalent cations through this putative channel are about 2.5–3.7 kcal/mole, comparable to the barriers observed electrophysiologically. This arrangement of amphipathic helices also indicates at least three potential energy minima, or stable positions for cations within the channel. X-ray diffraction studies in which terbium ions, a calcium analogue, can be localized using anomalous dispersion (Fairclough et al 1985) clearly identified three major locations for terbium within the transmembrane region. These observations support the view that the ion channel is occupied by cations even in the resting state, at least in vitro.

Closing of the channel must involve a diameter decrease below 7.0 Å. The event that closes the ion channel is currently open to conjecture, and some models present themselves as testable candidates. For example, the aromatic Phe and Tyr residues at position 443 could serve as a possible gate near the cytoplasmic end of the channel. Terbium-binding experiments showed that the channel is filled with cations through most of its length (Fairclough et al 1985) and suggested that a site 17 Å from the cytoplasmic face, where essentially no terbium is bound, is a likely location for the gate.

Conclusions

The sequences of the subunit chains in the AChR provide the essential map for localization of functional determinants. When the three-dimensional structure at atomic resolution emerges, sites mapped in the structure can be related to their effect upon channel opening or desensitization. This correlation requires a profound understanding of the physiological function, the chemical change, and the electrophysiological consequences of modulation. Each facet of this analysis demands the ultimate of forefront technologies, but in the end, the synthesis of these perspectives will yield the best understanding of any element in the neuromuscular synapse and probably of any protein involved in cell-to-cell communication.

Acknowledgments

We thank Geri R. Gilbert for typing the many versions of the manuscript, and Robert Love, Paul Bash, and Alok Mitra for their helpful suggestions and discussion. Related studies are supported by National Institute of Health Grant GM24485 and National Science Foundation Grant PCM83-16401 to R. M. S., National Institute of Health Grants GM09827 to M. P. M., and NS07241 to E. F. Y. Salary support for J. P. E. derives from National Institute of Health Grant NS13050 to Dr. Mark G. McNamee. S. C. was supported by a university fellowship from the University of California, Berkeley.
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Literature Cited

Adams, P. R. 1975. An analysis of the dose-response curve at voltage-clamped frog endplates. Pflegers Arch. 360: 145–53
Aslanian, D., Heidmann, T., Nigrie, M., Changeux, J.-P. 1983. Raman spectroscopy of acetylcholine receptor-rich membranes from Torpedo marmorata and of their isolated components. FEBS Lett. 164: 393–400
Cartaud, J., Sobel, A., Rousselet, A., Devaux,


Damle, V. N., Karlin, A. 1978. Affinity labeling of one of two α-neurotoxin binding sites in acetylcholine receptor from Torpedo californica. Biochemistry 17: 2039–45


Delegueane, A. M., McNamee, M. G. 1980. Independent activation of the acetylcholine receptor from Torpedo californica at two sites. Biochemistry 19: 890–95


Dunn, S. M. J., Conti-Tronconi, B. M., Raftery, M. A. 1983. Separate sites of low and high affinity for agonists on Torpedo californica acetylcholine receptor. Biochemistry 22: 2512–18


Guy, H. R. 1984. A structural model of the acetylcholine receptor channel based on partition energy and helix packing calculations. Biophys. J. 45: 249–61


Haring, R., Kloog, Y. 1984. Multiple binding sites for phencyclidine on the nicotinic acetylcholine receptor from Torpedo ocel-lata electric organ. Life Sci. 34: 1047–55


Heidmann, T., Changeux, J.-P. 1979. Fast kinetic studies on the allosteric inter-

actions between acetylcholine receptor and local anesthetic binding sites. Eur. J. Biochem. 94: 281–96

Heidmann, T., Changeux, J.-P. 1984. Time-resolved photolabeling by the noncompe-
titive blocker chlorpromazine of the acetylcholine receptor in its transiently open and closed ion channel conformation. Proc. Natl. Acad. Sci. USA 81: 1897–1901

Heidmann, T., Oswald, R. E., Changeux, J.-P. 1983. Multiple sites of action for non-
competitive blockers on acetylcholine receptor-rich membrane fragments from Torpedo marmorata. Biochemistry 22: 3112–27


Heuser, J. E., Salpeter, S. R. 1979. Organization of acetylcholine receptors in quick-


Kaldany, R. R., Karlin, A. 1983. Reaction of quinacrine mustard with the acetylcholine receptor from Torpedo californica. J. Biol. Chem. 258: 6263–42

Kaneda, N., Tanaka, F., Kohno, M.,
Hayashi, K., Yagi, R. 1982. Change in the intrinsic fluorescence of acetylcholine receptor purified from *Narke japonica* upon binding with cholinergic ligands. *Arch. Biochem. Biophys.* 218: 376–83


Moore, H. P., Raftery, M. A. 1979. Ligand-induced interconversion of affinity states in...
membrane-bound acetylcholine receptor from *Torpedo californica*. Effects of sulf-hydryl and disulfide reagents. *Biochemistry* 18: 1907–11


Oswald, R. E., Changeux, J.-P. 1981a. Selective labeling of the δ-subunit of the acetylcholine receptor by a covalent local anesthetic. *Biochemistry* 20: 7166–74


Interacting surfaces of neurotoxins and acetylcholine receptor. *Toxicon* 20: 83–93


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