Acetylcholine Receptor: An Allosteric Protein

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Summary. The nicotine receptor for the neurotransmitter acetylcholine is an allosteric protein composed of four different subunits assembled in a transmembrane pentamer $\alpha_2\beta\gamma\delta$. The protein carries two acetylcholine sites at the level of the α subunits and contains the ion channel. The complete sequence of the four subunits is known. The membrane-bound protein undergoes conformational transitions that regulate the opening of the ion channel and are affected by various categories of pharmacologically active ligands.

In 1906, Langley (*1*) concluded his experiments on the effects of nicotine on muscle contraction in the fowl by postulating that a "receptive substance . . . combines with nicotine and curari and is not identical with the substance which contracts." Sixty-five years later, the nicotinic receptor for acetylcholine was isolated (*2*) as the first in a still-increasing list of receptors for neurotransmitters (*3*) and remains today the only one for which complete primary structure data have been obtained via the application of complementary DNA (cDNA) cloning and sequencing techniques (*4–11*). Several factors contributed to such a rapid progress: (i) the use of the electric organ from the fish *Electrophorus* or *Torpedo*, a tissue exceptionally rich in a single class of cholinergic synapse (*12*); (ii) the availability of the small polypeptide α-toxins from snake venom, highly selective labels of the acetylcholine binding site (*2, 13*); and (iii) the hypothesis that this site should be associated with a protein (*12*) and resemble the regulatory site of an allosteric protein (*14, 15*).

The research on the acetylcholine receptor developed in several successive steps, beginning with its characterization in detergent extracts (*2, 15*) and its purification from *Electrophorus* (*16–19*) and *Torpedo* electric organs (*20*). Its subunit composition (*21–24*) and the partial primary (*24, 25*) structure of its subunits were later identified, the purified molecule was reconstituted in a functional state into artificial lipid vesicles (*26, 27*) and planar bilayers (*28*), and the structural transitions that account for the regulation of the associated ionic channel were analyzed (*29–34*). As a consequence, the acetylcholine receptor has become the most well-characterized receptor for a neurotransmitter and also one of the most thoroughly investigated membrane proteins with unambiguous allosteric properties (*15, 35–41*). Finally, basic studies on this molecule led to the development of an experimental model of the human disease, myasthenia gravis (*42, 43*).

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Morphology of the Receptor Protein

The initial work (*15*) on the characterization of the acetylcholine receptor from detergent extracts of *Electrophorus* and *Torpedo* electric organs revealed an α-toxin binding component with a 9*S* sedimentation coefficient and a 70-Å Stokes radius. The molecular weight of this component (*15, 23, 44*) has been estimated to be 292,000 (292K) to 303K from laser scattering data (*45*) and radiation inactivation (*46*), in close agreement with the values of 285K to 290K computed from the amino acid sequences of the subunits (deduced from cDNA clones) (*7–11*) and the covalently linked sugars (~20K) (*47*). This light form, which carries two sites for α-bungarotoxin (*23*), binds significant amounts of detergents [up to 0.4 g per gram of protein (*44*)] and has a nonglobular shape characterized by a radius of gyration of 46 ± 10 Å given by low-angle neutron scattering (*44*).

In crude and purified preparations of *Torpedo* receptor, a second 13*S* α-toxin binding component (*23, 48*) with a Stokes radius of 85 Å and a molecular weight exactly twice that of the light form (*23*) coexists with the light form in variable ratios. Reducing agents, such as 2-mercaptoethanol or dithiothreiotol, convert this heavy form in vitro to a state indistinguishable from the light form (*15, 35, 40*). The heavy form thus represents a dimer of two light forms linked covalently by an intermolecular disulfide bridge.

The denaturing detergent sodium dodecyl sulfate (SDS) dissociates the receptor protein from electric organs of fish and muscle tissue of all vertebrate species studied into four different polypeptide subunits that migrate on SDS gels with apparent molecular weights (*21, 22*) of 39K (α), 48K (β), 58K (γ), and 64K (δ) (*15, 35, 36*). The stoichiometry of the chains within the 290K light form, originally inferred from molecular weight measurements (*23*) and definitively established by both preparative gel electrophoresis in SDS (*49*) and cosequencing (*24*) of the subunits, is $2\alpha \cdot 1\beta \cdot 1\gamma \cdot 1\delta$. The receptor oligomer thus has a rather uncommon heterologous pentameric organization.

Solubilization of the receptor oligomer requires the dispersion of the membrane by nondenaturing detergents (*2*). It is an integral membrane protein. Selective proteolysis (*50–52*), antibody binding (*53–59*), and radioisotopic iodination (*60, 61*) of closed and open membrane vesicles further show that its five constitutive polypeptide chains traverse the membrane.

Electron microscopy of negatively stained, purified, and membrane-bound preparations of receptor disclose ringlike particles or rosettes, 80 to 90 Å in diameter, with a stain-filled central pit (*38, 62–66*) (Fig. 1A). Each of the rosettes represents a frontal view of a light form (*67*). Computerized image analysis at a resolution of 17 to 18 Å (*68*) reveals, in agreement with the known stoichiometry of the chains, five unequal peaks of electron density distributed around the central pit (Fig. 1C). The precise assignment of each of these peaks to specific subunits is not definitely settled except for the α subunit, which carries at least part of the α-bungarotoxin binding site. The two α chains, identified by biotinylated

α-toxin (*69, 70*), image analysis (*68, 71*), or monoclonal antibody fragments (*66*), have been reported to make angles of 110° ± 30° (*69, 70*), 144° ± 4° (*66*), or 160° (*68*) and thus are not adjacent within the oligomer. On the basis of cross-linking experiments, the order α γ α δ β (*36, 69, 70*) has been proposed [see however (*64*)].

When viewed on the side (Fig. 1B) (*38, 63, 65*), the light-form molecule appears roughly as a cylinder (110 Å long) with an axial well (7.2 Å in diameter) filled with stain, which may represent the ion channel (*65, 66*). It extends above the lipid bilayer by 45 to 55 Å into the synaptic cleft and by about 15 to 20 Å on the cytoplasmic side (*65, 66, 72*). Small-angle x-ray diffraction studies provide evidence for an extension of electron density of 7 to 8 Å above the top of the synaptic head of the receptor associated with α-toxin binding, suggesting an apical location of its sites (*66*). The receptor molecule thus appears as a roughly cylindrical bundle with a transverse polarity and subunits arranged like staves around a rotational axis perpendicular to the plane of the membrane (*15, 64, 65, 73*) (Fig. 1B).

Both in purified preparations or in situ, the heavy form looks, in agreement with the molecular weight studies (*23, 46*), like a pair or doublet of light-form rosettes (*68, 74, 75*) (or, on the side, cylinders) juxtaposed at a center-to-center distance of 90 to 95 Å (Fig. 1A). The relative orientation of the two rosettes in a doublet, investigated by image analysis of negatively stained (*68*) or antibody-labeled (*66*) preparations, varies [see however (*76*)] with a privileged side-by-side (R · R) disposition (*64*), indicating a flexibility of the cross-link between the two light forms.

Binding Sites for Pharmacologically Active Ligands and Functions of the Subunits

Agonists and competitive antagonists binding sites. The purified and membrane-bound receptor carries two primary acetylcholine binding sites to which the nicotinic agonists and antagonists and the snake venom α-toxins attach in a reversible and mutually exclusive manner and with the rank order expected from their pharmacological action (*15, 35, 39, 77*).

This pair of sites has been covalently labeled by derivatives of cholinergic effectors such as *p*-(trimethylammonium) benzene diazonium fluoroborate (TDF) (*78, 79*) and a closely related compound 4-(*N*-maleimidophenyltrimethyl ammonium) (MBTA) (*36, 80*), which both behave as irreversible antagonists. In the case of MBTA, however, as with a series of related compounds (*36*), the covalent attachment exclusively occurs after treatment by an agent that breaks disulfide bonds (dithiothreiotol), thus exposing a reactive sulfhydryl located close to the negatively charged subsite of the acetylcholine binding site (*36*). Other affinity labeling reagents—such as bromoacetylcholine (*81*) and the photoisomerizable 3-(α-bromomethyl)3′-(trimethylammoniummethyl)azobenzene (*82*)—exert an agonistic, α-toxin–sensitive action when they bind covalently. This finding supports the conclusion that the sites labeled are those involved in the regulation of ion permeability. The same sites are, of course, tagged by snake venom α-toxins after derivatization (*83*), addition of cross-linking agents (*84*), or simple ultraviolet irradiation (*85*).

As is commonly found with typical regulatory proteins (*86, 87*), the equilibrium binding curve of acetylcholine (and other cholinergic ligands) to these sites on the membrane-bound (*77, 88–91*) or purified (*92*) receptor exhibit a sigmoid shape [Hill coefficient (n_H), 1.3 to 1.4]. The two primary sites of the light-form oligomer thus interact in a positively cooperative manner. However, surprisingly, these two sites do not exhibit perfectly symmetrical properties and differ, in particular, in their kinetic parameters of the interaction with the snake α-toxins (*88, 93, 94*), ^{3}H-labeled MBTA, and ^{3}H-labeled bromoacetylcholine (*95*); by the equilibrium binding constants of *d*-tubocurarine (*77, 96*); and by the sensitivity to irreversible inactivation by the coral diterpenoid lophotoxin (*97*).

The affinity labeling reagents and α-toxin compounds, which selectively block these sites, labeled the α subunit (*21, 36*) with or without prior reduction by dithiothreiotol. Also, the α subunit isolated in SDS still bound α-bungarotoxin (*98*). A perfect agreement exists between the presence of the two primary acetylcholine binding sites and the presence of two α chains per light-form pentamer (Fig. 1D). The α chains therefore carry at least part of these sites. Since the two α chains are not adjacent, the cooperative interactions between acetylcholine sites are thus indirect or allosteric. The contribution of other subunits to binding of the rather large snake venom α-toxins has been shown under various conditions of covalent attachment (*83–85, 99*).

Binding sites for noncompetitive blockers. In both the native membranes and reconstituted (*100*) receptor, the ionic response is inhibited by a class of particularly potent pharmacological agents, the noncompetitive blockers, which are postulated to interfere directly or indirectly (or both) with the ion channel (*101, 102*). The light-form pentamer has sites for these pharmacological agents that have been analyzed with a fluorescent ligand [quinacrine (*34*)] and various isotopically labeled derivatives of local anesthetics (*103, 104*) of the frog toxin perhydrohistrionicotoxin (*105*), of the hallucinogen phencyclidine (*104, 106*), and of the neuroleptic chlorpromazine (*104, 107*). Under equilibrium conditions, these noncompetitive blockers reversibly enhance (to different degrees) the binding of cholinergic ligands to the acetylcholine receptor site, converting the shape of their binding curves from a sigmoid to a hyperbola (*89*). Conversely, cholinergic ligands potentiate their binding (*34, 103, 104*). Reciprocal affinity interactions take place between the primary acetylcholine binding sites and the sites for the noncompetitive blockers, which are thus viewed as allosteric binding sites (*86*). These have been subdivided into two main categories (*104*): (i) high-affinity sites, sensitive to histrionicotoxin and present as a unique copy per receptor pentamer; and (ii) low-affinity sites, insensitive to histrionicotoxin and much more numerous (10 to 20 times the number of α-bungarotoxin sites) and lipid dependent, that is, most likely to be located at the interface of the receptor with membrane lipids.

Early attempts to identify the chain or chains of the receptor involved in the high-affinity site for noncompetitive blockers were done with *T. marmorata* receptor and an azido derivative of the potent local anesthetic trimethisoquin. Only the δ chain incorporated the label in an agonist-dependent manner (*107, 108*). Both ^{3}H-labeled perhydrohistrionicotoxin and phencyclidine, under ultraviolet irradiation (*107*), gave similar results. However, with *T. californica* and quinacrine mustard (*36, 109*) [or triphenylmethylphosphonium (*110*)] or with *T. ocellata* and azidophencyclidine (*111*), labeling occurred primarily at level of the α or the β chains, respectively. With *T. marmorata*, ^{3}H-labeled chlorpromazine labeled all four chains, suggesting that they all contribute to the single high-affinity site present per oligomer (*104, 107*). The most probable position for such a site is obviously the central hydrophilic crevice visualized by electron microscopy (Fig. 1D), where the distances to all five chains are minimum. Minor sequence variations (*11*) would then probably account for the differences

between *Torpedo* species (*112*). A rather similar location for an allosteric site has been described for diphosphoglycerate in hemoglobin (*113*).

Role of the δ *chains*. Finally, it is at the δ chain (*114, 115*) where the disulfide link between the two light forms in a heavy-form dimer occurs at a cysteinyl residue located near its carboxyl-terminal end (*116*).

Chemistry and Molecular Genetics

The gross chemical properties of the purified receptor protein, such as its average amino acid composition, state of glycosylation, and isoelectric point, did not reveal striking features except, possibly, a hydrophobic character slightly higher than that of most globular proteins (*15*). In contrast, for a regulatory protein an asymmetrical pentameric organization from four subunits differing by their molecular weight and peptide map (*117*) looked unconventional. The development of monoclonal antibodies to each individual chain first revealed extensive immunological cross reactions between subunits (*118*). Microsequencing of the purified subunits (*24, 25*) then showed important sequence homologies (*24*) between the first amino-terminal amino acids of the polypeptide chains, leading to the proposal that the $\alpha_2\beta\gamma\delta$ oligomer may, indeed, have some hidden symmetry (*15, 35*). The forceful entry of molecular genetics, while confirming these views, opened a new era by the complete deciphering, in less than a year, of the entire primary structures of the four receptor subunits.

About 2.4 percent of the total messenger RNA (mRNA) in the electric organ codes for the receptor polypeptides (*119*), and therefore it was used to prepare cDNA libraries from which clones coding for the receptor subunits have been successfully selected by two methods: (i) screening for electric organ specificity, selection of mRNA on those clones by the hybridization-selection technique, and identification of the products obtained in vitro by immunoprecipitation (*4, 5*); and (ii) direct hybridization with two different sets of oligodesoxyribonucleotides corresponding to known fragments of subunit sequences (*6–9*).

Complementary DNA clones coding for the α chain of *T. marmorata* receptor (*5, 6*) and for the α (*7*), β (*8*), γ (*4, 9*), and δ (*8*) chains of *T. californica* receptor have been isolated. The mRNA species encoding them are about 2000 base pairs for the α (*5–7*), β (*8*), and γ (*4, 9*) subunits and about 6000 base pairs for the δ subunit (*8, 120*). The mRNA's are thus much larger than expected from the length of the coding sequences, an indication of the presence of long untranslated regions. Partial (*6*) and complete (*11*) nucleotide sequences for the precursor of the α chain in *T. marmorata* and the complete sequence (Fig. 2) coding for the α (*7*), β (8), γ (*9, 10*), and δ (*8*) chains in *T. californica* have been established, and the corresponding amino acid sequences have been deduced. These se-

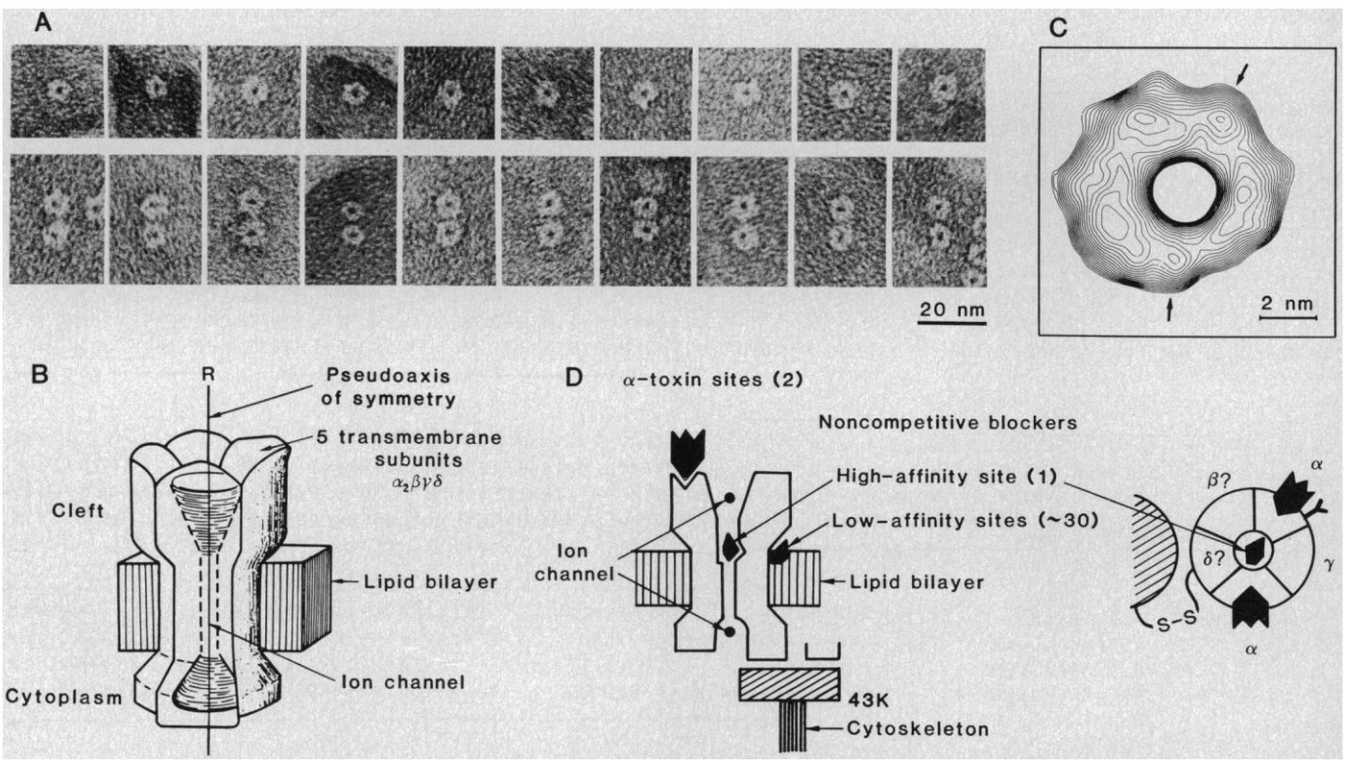


Fig. 1. Morphology and binding sites of the acetylcholine receptor from *Torpedo* electric organ. (A) Electron micrographs of the 290K pentameric ($\alpha_2\beta\gamma\delta$) light form and of its heavy-form dimer viewed from the top. [Courtesy of J. Cartand (*24*)] (B) Schematic model of the light-form pentamer viewed on the side. The suggested distribution of protein masses across the lipid bilayer is based on neutron scattering (*44*) and amino acid sequence data (*7–11*). (C) Average image of the light-form pentamer given by computerized image analysis of electron micrographs such as those of (A). The two peaks indicated by an arrow are reinforced by α-bungarotoxin and thus probably correspond to the α subunits (*229*). [Courtesy of the *Journal of Molecular Biology* (*68*)] (D) Schematic distribution of the binding sites for pharmacologically active ligands on the light-form pentamer. (Left) (i) The two primary sites for acetylcholine and snake venom α-toxins have an apical location (*66*) on the α subunits; (ii) the unique high-affinity site for noncompetitive blockers is located in or near the ion channel; (iii) multiple low-affinity sites for noncompetitive blockers are distributed at the lipid-protein interface; (iv) association with the peripheral protein (43K) on the cytoplasmic face immobilizes the receptor molecules in the membrane by cross-linking with the cytoskeleton. (Right) The top view of the pentamer shows the arrangement of the subunits around the central ion channel (*36*). Localization of the β and δ subunits in the rosette are still debated. The proposed differences in glycosylation state of the two primary acetylcholine binding sites on the α subunits (*131*) is schematically represented by a Y. The δ chains link the two light forms of a heavy-form dimer in a flexible manner.

quences include the amino-terminal peptide sequences formerly identified with the purified α chain from *T. marmorata* (*25*) and α, β, γ, and δ chains from *T. californica* (*24*). They are preceded by a signal peptide (*121*) of 24 (α and β), 17 (γ), and 21 (δ) amino acids, largely hydrophobic, that are absent in the mature subunits. The coding sequences of the mature subunits in *T. californica* are 437 (α), 469 (β), 489 (γ), and 501 (δ) amino acids long with exact molecular weights of 50,116 (α), 53,681 (β), 56,279 (γ), and 57,565 (δ). As already noticed with the partial amino acid sequence data (*24*), homology between chains ranges, depending on the region, from 10 to 60 percent amino acid sequence identity with an average of 40 percent. The close homology found between α and β on one hand and between γ and δ on the other suggests a phylogenetic tree from a single ancestral gene (*24*) with a first branching for the α and β chains and a second branching for the γ and δ chains (*9*).

Translation in vitro of each receptor subunit mRNA requires the presence of a formylmethionine transfer RNA (tRNA), indicating that each is translated from a discrete and separate mRNA (*121*). It occurs on membrane-associated polysomes and involves the cleavage, soon after synthesis, of the already mentioned amino-terminal signal sequences (*121*). Neither α-bungarotoxin binding nor the exact assembly of the four subunits into the pentameric light form has been achieved in these cell-free systems. However, these processes occur in the mouse muscle cell line BC3H1 (*122, 123*) during the 2 hours subsequent to the synthesis of the receptor. Only 1 minute, however, is needed for the polymerization of the amino acids, membrane insertion, and cotranslational core glycosylation at one or several asparagine residues in the amino-terminal region of the chains (*57, 121–123*). During the succeeding 15 to 30 minutes, the α subunit acquires the ability to bind α-bungarotoxin but is still in a monomeric (5*S*) form (*122, 123*). Tunicamycin blocks the conversion to this α-toxin binding state, indicating that glycosylation is necessary at this step (*122, 123*). After this conformational maturation, the subunits directly assemble into the heterologous oligomer, and covalent attachment of lipids is required for this process (*124*). Only one-third to one-fourth of the total amount of the synthesized subunits (at least for α and β) persists in the mature protein, the rest being degraded at a very fast rate (half-life, 0.5 hour) (*122, 123*). After transit, possibly in a vesicular form (*125*), from the endoplasmic reticulum to the various subcompartments of the Golgi apparatus, acetylcholine-regulated ion channels appear on the surface of the cell.

Functional acetylcholine channels

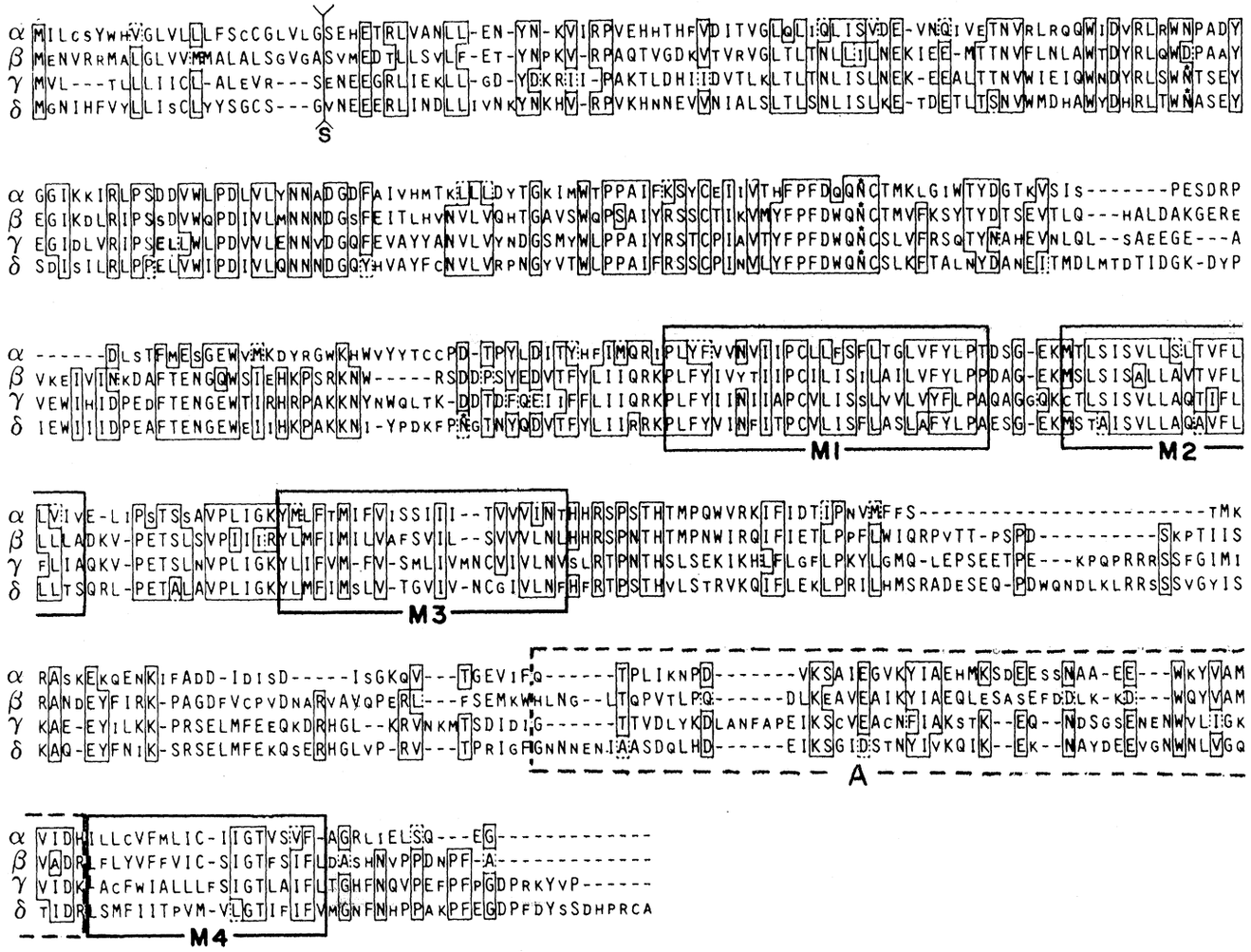


Fig. 2. Amino acid sequences (deduced from cDNA clones) of the α, β, γ, and δ subunits from *Torpedo californica* (*7–11*) aligned as described (*9*). Amino acid positions identical in at least two of the subunits are enclosed with solid light lines. An amino acid subtituted at one position by a residue with an equivalent hydrophilic versus hydrophobic character is enclosed in a light interrupted line. The four transmembrane α-helices are enclosed by a heavy line. The "amphipatic" helix A (*66, 181, 182*) is marked by a dark dashed line. S indicates the cleavage site of the signal peptide.

have been recorded by electrophysiological methods in *Xenopus* oocytes after injection of total mRNA from *Torpedo* (*126*) or from denervated muscle (*127*). The same result was achieved by injecting the four subunit-specific mRNA's obtained by transcription of the cDNA's in expression vectors (*128*). The vectors contained SV40 (simian virus 40) early gene promoter, donor, and acceptor splice sites derived from the rabbit β-globin gene and were transcribed individually in subunit-specific mRNA by transfection of permissive monkey cells (Cos), enriching the yield 0.9 to 6.8 times above their amount in *T. californica* polyadenylated RNA. The mixture of the four specific mRNA's directed the synthesis of a membrane-bound receptor channel complex indistinguishable from the normal receptor. Deletion of the α chain–specific mRNA expectedly abolished α-bungarotoxin binding and the ionic response. Deletion of the mRNA coding for any one of the other subunits preserved the binding of α-toxin (although to a smaller extent than in the complete mixture). This deletion almost completely abolished the electrical response (a few responsive oocytes were, however, obtained with the αβγ and αβδ combinations but with a reduced sensitivity), a result leading to the conclusion that the presence of the four subunits is required for a fully functional receptor.

Another outcome of the molecular genetics approach bears on the previously mentioned, although unexplained, asymmetry between the two primary acetylcholine sites present per oligomer. One possibility was that the two α chains have different sequences and are thus coded by different chromosomic genes. Southern blot hybridization of genomic DNA with specific cDNA probes supports the existence of a single gene for the α chain in *Torpedo* (*129*) and in the mouse (*122, 123*) and for the δ chain in *Torpedo* (*120*). The difference in stoichiometry of the α and βγδ chains in the mature oligomer thus results from post-transcriptional events. The most probable interpretations for the different properties of the two primary acetylcholine sites are thus a difference of subunit environment or a post-translational modification such as glycosylation (*130, 131*); both possibilities can be accounted for by studies with monoclonal antibodies (*94*).

Complete coding sequences of receptor subunits have been deduced from cDNA or genomic clones for calf α subunit (*132*) [for which partial amino-terminal sequences had already been determined chemically (*133*)], calf γ subunit (*134*), and human α subunit (*132*), and partial sequences have been determined for chick (*135*) and mouse (*123*) α chains. The α subunits from the different species show great similarities. Human and calf α chains, like that of the *Torpedo*, have 437 amino acids and show respectively 81 and 80 percent identity with the α subunit from *T. californica* (*132*). The γ subunits of calf and *Torpedo* (*134*), in contrast, show only 56 percent amino acid homology, possibly because of a lesser contribution of the γ chain to the receptor function. The structure of the acetylcholine receptor protein thus appears to be exceptionally well conserved throughout the vertebrate phylum, and one could almost say that what is true for the *Torpedo* receptor is also true for the human receptor.

Channel Opening and Acetylcholine Binding

Signal transmission at the neuromuscular junction and the electromotor synapse takes place within milliseconds and involves the fast release (microseconds) of a pulse of acetylcholine, the local concentration rising transiently to 10^{-4} to $10^{-5}M$ in the synaptic cleft (*136, 137*). Our understanding of the mechanism by which this acetylcholine pulse opens the ion channel has significantly increased since the development of systems in vitro with which both binding and ion flux can be monitored simultaneously in the time scale of milliseconds. These systems include purified, extrasynaptic membranes from *E. electricus* (*29*), receptor-rich subsynaptic membranes from *Torpedo* (*138*), and purified, functionally reconstituted receptor incorporated into liposomes (*27*). In all these models, tightly closed vesicles or "microsacs" form, with which acetylcholine-regulated transport of tracer ion can be measured in the time scale of milliseconds to seconds by quench flow and rapid filtration methods with radioactive (*37, 139, 140*) or nonradioactive (*141*) permeant ion, by quenching with heavy permeant cations such as thallium (*142*) or cesium (*37*), or by the fluorescence emission of a dye like anthracene-1,5-disulfonic acid entrapped in the vesicles.

Channel opening. Taking the initial rates of ion transport (influx or efflux) as an index of channel opening (*37, 139, 140, 143*), the dose-response curves (*37, 141, 143*) closely resemble those established by electrophysiological recording under fast electrophoretic release of agonist (*144, 145*). The half-maximum response occurs at the corresponding concentrations of 10 μM suberyldicholine, 45 to 80 μM acetylcholine, and 300 to 800 μM carbamylcholine; the maximum responses follow the same rank order: suberyldicholine > acetylcholine > carbamylcholine (*37, 140, 146*). This maximum value depends on the number of open channels and on an intrinsic rate constant J (*37*), which corresponds to the intrinsic conductance, γ, of the open channel (*147*). As was found in "noise" and single channel measurements, this constant is independent of the nature of the agonist (*37, 142, 147–149*) and yields in both series of data similar estimates of 10^6 to 10^7 ions transported per second and per open channel (*142–149*). The agonist-specific differences in the maximum number of open channels thus result from variations in the channel-opening isomerization constant (*37, 141*) and probably because of differences in the rate of channel closing (*145, 149*).

As has been found with many allosteric proteins (*86, 87*), and in agreement with the early recordings of the physiological response (*144, 145, 150*), the shape of the agonist dose-response curves appears to be systematically sigmoid (n_H, 1.8), suggesting that a minimum of two agonist molecules contributes to channel opening. This interpretation is consistent with the presence of two primary acetylcholine binding sites per light form that cooperatively interact under equilibrium binding conditions (*151*).

Selective blocking of one of them by α-bungarotoxin (*90*) or by the covalent affinity label MBTA (*152*) results in a large decrease by a factor of at least 20 in the initial rate of agonist-dependent ion transport, confirming that channel opening requires a strong cooperation between the two primary binding sites (*153*).

The establishment of an ionic gradient across the microsac membrane creates conditions of voltage clamp under which the dose-response curve for acetylcholine changes with electrical potential. At −45 mV and high acetylcholine concentrations (above 300 μM), the initial flux decreases, indicating (*154*), in agreement with electrophysiological data (*153, 155*), a voltage-dependent blocking of the ion channel by acetylcholine.

Prolonged application of agonist on the isolated membranes or reconstituted receptor from *Torpedo* (*143, 156, 157*) and *Electrophorus* (*37, 139, 146*) results, as initially observed in electrophysiological experiments (*150, 158, 159*), in a time-dependent reversible decrease of the response called desensitization. This phenomenon comprises two processes

(*158, 160, 161*): a fast one at the rate of 2 to 7 per second (*158, 161, 162*) up to 75 per second (*141*), and a slow one at the rate of 0.01 to 0.1 per second (*141, 143, 162*), except in *Electrophorus* where only the fast one has been observed (*37*). The rapid phase leads to a thousandfold decrease in initial flux, the slow one to undetectable ion transport (*162*). The relative amplitude of the two phases obtained by recovery measurements depends on the time of exposure to the agonist, an indication that the processes are interdependent (*161*). The rate of the rapid phase varies in a cooperative manner with agonist concentration (*141, 163*), while that of the slow phase does not (*141, 143*). At low concentrations of agonist (*143*), complete desensitization can occur without significant activation.

Rapid agonist binding. The early attempts to relate agonist binding and opening of the ion channel initially performed with membrane fragments, although the same holds for reconstituted vesicles, met with a puzzling paradox (*89*). The dissociation constant for acetylcholine measured at equilibrium approached 10 n*M* (*88*), about 4 to 5 orders of magnitude below the apparent dissociation constant for the permeability response. Assuming that the association rate for acetylcholine is diffusion-controlled, its dissociation from such high-affinity sites would occur in 10 to 100 msec, during which time the synapse would be blocked. Accordingly, high-affinity binding could not cause channel opening.

Examination of the evolution of receptor binding properties as a function of time after agonist mixing resolved the paradox (*30, 32*). Acetylcholine and other cholinergic ligands, including some agonists, shift the receptor from a state of low-affinity binding to a high-affinity state at equilibrium. The various successive steps in the process have been resolved by rapid mixing techniques with (i) the fluorescent agonists dansyl-6-choline (*164*) or NBD-acetylcholine (*39, 165*), (ii) isotopically labeled cholinergic ligands and rapid filtration (*166*), and (iii) changes of intrinsic (*33*) or extrinsic fluorescence after covalent modification of the receptor with a fluorescent probe (5-iodoacetimidosalycilic acid) (*167*). Each method has its drawbacks, including uncertainties about the quantum yield for (i) and (iii), time limitation for filtration for (ii), and alterations of receptor properties for some in (iii). Studies with dansyl-6-choline (*164*) and [^{3}H]acetylcholine (*166*) show that 20 percent of the receptor exists in a state of high affinity (3 n*M* for acetylcholine and 2 n*M* for dansyl-6-choline in the absence of agonist (*168*), the remaining 80 percent exhibiting a low affinity for agonists. Beyond the details of the analysis, which are often linked to a particular kinetic model, two major conformational transitions (*164, 167, 169*) have been resolved. These are a slow one, with an apparent rate constant (k) of about 0.01 per second which increases to a plateau (*164*) or decreases after reaching a maximum (*167*) with agonist concentration; and an intermediate one, with a k of 2 per second (*167*) to 50 per second (*169*).

The state stabilized after equilibration binds acetylcholine and dansyl-6-choline with respective dissociation constants of 3 n*M* and 2 n*M*; in this respect it does not differ from that present as 20 percent of the population in the membrane before agonist binding [see however (*170, 171*)]. The state reached by the intermediate transition has an apparent equilibrium constant for agonists in the range of 1 μ*M*. Faster events corresponding to activation have not yet been resolved by the techniques utilized, with some exceptions (*151*).

Channel opening and agonist binding. Comparison of the rapid kinetic measurements of agonist binding and permeability response in the same time scale and with the same membrane preparation revealed that the slow transition coincides exactly with the slow desensitization phase of the ionic response (*141, 143*) and that the intermediate transition monitored with dansyl-6-choline fits with the fast desensitization (*141*). These experiments further showed that the initial rate of agonist-dependent ion transport and the apparent rate constant of the intermediate relaxation process vary in parallel with agonist concentration (*141*); since both parameters are directly proportional to the low-affinity activatable conformation of the receptor, it can be inferred that this low-affinity binding causes the opening of the ion channel.

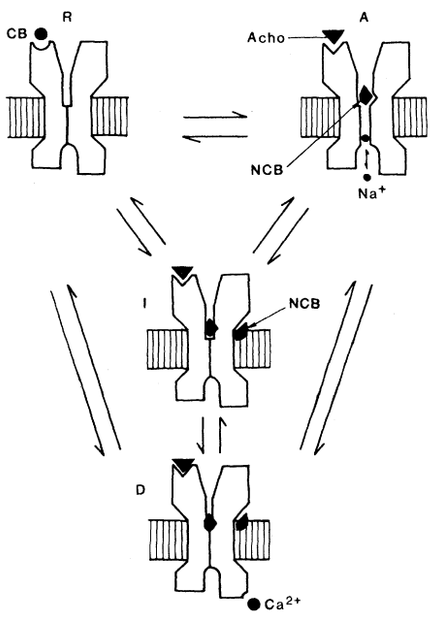
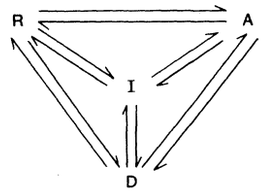


Fig. 3. Minimal four-state model for the allosteric transitions of the acetylcholine receptor.

Model. The simplest minimal model (*140, 169*) compatible with these data is an adapted version of the concerted model for allosteric transitions (*87*) and of that proposed by Katz and Thesleff (*150*) for desensitization (Fig. 3). Here, A



corresponds to the active state with the channel open, and I and D correspond to rapidly and slowly desensitized states, respectively. The same two primary acetylcholine sites are involved in each state, but their affinity increases from R to D via A and I. All these states are discrete, interconvertible, and, for some of them, present before ligand binding. Their respective dissociation constants are at least 50 to 100 μ*M* (R), less than 1 μ*M* (I), and 3 to 5 n*M* (D) for acetylcholine (*166*) and dansyl-6-choline (*164, 169*). During transmission, the local concentration of acetylcholine is about 5 to 10 times the K_d for the R state, yielding nearly saturation of the low-affinity state (*136*). Interestingly, the dissociation constant for the desensitized D state fits with the nonquantal "leak" concentration of acetylcholine measured at the level of the postsynaptic membrane in the presence of esterase inhibitors (*137*).

In agreement with this model, a significant fraction of the D state is detected in reversible equilibrium with the resting conformation before agonist binding, and the openings of the ion channel follow all-or-none transitions. However, substates have been reported for both the high-affinity (*171*) and the active state (*153, 172*). Also, the two acetylcholine binding sites cooperate positively but exhibit distinct properties that may create ordering in the binding of acetylcholine (*165*). All these features should be included in a complete scheme.

Consequences of the model. The action of the noncompetitive blockers on flux and binding kinetics provides further support to the allosteric four-state model. In agreement with the many re-

ports of electrophysiologists (*101, 102, 173*), rapid ion flux measurement with *Torpedo* microsacs shows that the noncompetitive blockers, such as procaine, cocaine, and phencyclidine, decrease the initial ion flux (*174*), a finding that is consonant with the hypothesis of direct blocking of the ion channel. However, these compounds could also increase the rate of fast desensitization of the ionic response (*173, 174*). Rapid binding measurements of agonists show that, at equilibrium, the noncompetitive blockers tested shift the receptor toward the D state to greater or lesser extents depending on the structure of the compound and its relative affinity for the R and D states. The stabilization occurs either via the histrionicotoxin-sensitive high-affinity site for noncompetitive blockers (meproadifen) or their multiple, low-affinity, lipid-dependent sites (trimethisoquin) (*104*). These blockers also accelerate the slow (*164, 170, 171, 175*) and intermediate (*104, 164*) transitions towards the D and I states, respectively. Conversely, the cholinergic ligands affect, through similar allosteric mechanisms, the rapid interaction of fluorescent (*34, 176*) or isotopically labeled noncompetitive blockers to the receptor, the association rate constants of perhydrohistrionicotoxin (*177*) and of phencyclidine (*178*) increasing by a factor of 10^3 to 10^4.

Rapid mixing experiments carried out under conditions of covalent attachment of chlorpromazine (*179*) or quinacrine azide (*180*) show an increased rate (~1000 times faster) of labeling of the multiple chains which contribute to their high-affinity site. None of the competitive antagonists tested exert this effect, and desensitization reduces this enhanced labeling. The correlation observed between this fast labeling and channel opening further supports the hypothesis that these noncompetitive blockers bind to a site probably located within the channel, which becomes accessible when the channel opens.

The precise changes in tertiary or quaternary structure that occur during activation and desensitization remain to be explored. An eventual concerted "tilt" of the subunits, which changes the diameter of the channel while preserving their "cryptic" symmetry (*15, 181*), looks attractive but requires experimental demonstration.

Models of Transmembrane Organization

A condition for such hidden symmetry (*15, 35*) of receptor quaternary structure is that the component subunits present similar tertiary organization. A straightforward inspection of the aligned sequences of the four subunits (Fig. 2) makes this assumption reasonable (*9–11*). All show a similar nonuniform distribution of hydrophobic amino acids, which leads to a common subdivision of the chains into (i) the amino-terminal signal sequence cleaved off in the mature protein; (ii) a large hydrophilic domain of 210 to 224 amino acids; (iii) a composite hydrophobic region of 68 residues subdivided into three stretches of 19 to 27 amino acids bounded by charged residues and interpreted as transmembrane α-helices (numbered I, II, and III in Fig. 2) by analogy with known membrane proteins such as bacteriorhodopsin and glycophorin A; (iv) a small hydrophilic domain of 109 to 146 amino acids; and (v) a carboxyl-terminal segment of 29 to 46 hydrophobic residues, again interpreted as a membrane α-helix (IV).

A consensus exists about the inside-out disposition of the hydrophilic domains. Proteolytic attack from the cytoplasmic side of the membrane-bound receptor preserves the amino-terminal sequence and sugar residues of the mature subunits (*50*). The large hydrophilic domain thus faces the synaptic cleft, and the small hydrophilic one faces the cytoplasm. The large hydrophilic domain would then carry the glycosyl residues, the main antigenic determinants (*43*), and the primary acetylcholine binding site on the α subunit. An unusual tandem of cysteinyl residues, present at amino acid positions 192 and 193 of the α chain (*6, 7, 11*) and absent in the other subunits, are potential sites for attachment of the maleimide affinity labeling reagents of the acetylcholine site; cysteinyl at position 170 has also been considered (*7*), even though it has homologous counterparts in the other subunits. The differences in molecular weight between subunits would then become apparent on the small hydrophilic domain facing the cytoplasm.

The number and orientation of the transmembrane segments, as yet undetermined, is critical since it concerns the disposition of the "ionic" channel viewed in all cases as an "aqueous pore" (*182*) delimited by the quasisymmetrical arrangement of the same transmembrane segment from each subunit. According to one scheme (*9, 11*) (Fig. 4A), the chain traverses the lipid bilayer four times at

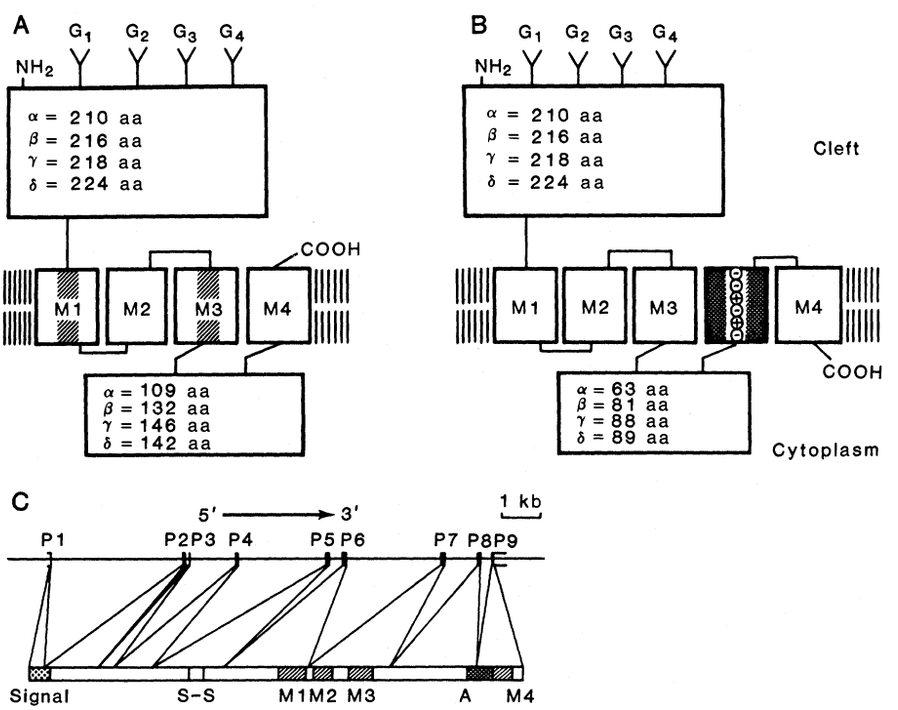


Fig. 4. Alternative models for the transmembrane organization of *Torpedo* receptor subunits inferred from their amino acid sequences (*9*). (A) Four transmembrane α-helices. Either helix I (*11*) or helix III (*9*) contribute to the uncharged ionic channel common to all five subunits. The carboxyl-terminal segments face the synapse. (B) Five transmembrane α-helices. An additional "amphipatic" helix A contributes to the charged ionic channel (*181, 183*). The carboxyl-terminal segments face the cytoplasm. The boxes facing the cleft and the cytoplasm have surfaces proportional to the protein masses. Numbers of amino acids in each domain are given inside the boxes. On the synaptic domains the carbohydrates are represented by the symbol Y. (C) A conspicuous similarity exists between the distribution of exons and introns in the human chromosomic gene coding for the α subunit and the models of transmembrane organization except for the putative helix A segment, which is split by an intron.

helices I to IV and, as a consequence, the carboxyl-terminal ends of the chains face the synaptic cleft. Helix I has been suggested as the component of an uncharged channel because of its highly conserved homology from one subunit to the other (*9*); alternatively, helix III has also been considered because of the vertical alignment of polar amino acid side chains in the middle of the helix, particularly evident on the α and β chains (*11*). The cationic selectivity filter for the channel would then be accounted for by the charged side chains from the extrinsic synaptic and cytoplasmic domains.

A second scheme (*181, 183*) (Fig. 4B) postulates an additional fifth transmembrane segment A formed at the expense of the cytoplasmic domain, thus reorienting helix IV to expose the carboxyl terminals to the cytoplasmic face. This model, which is based on computer probing for periodicities in hydrophobicity by Fourier analysis (*183*) or transfer-energy calculations (*181*), assumes that the additional transmembrane segment A makes an "amphipathic" helix with a continuously hydrophobic face on one side and a hydrophilic face on the other, yielding a cation-selective charged channel with 21 negative and 19 positive charges.

Distinction between the uncharged channel with four helices and the charged channel with five helices will result from the transmembrane mapping of critical peptides characteristic of each model and identified either by relevant monoclonal antibodies (directed, for instance, against the carboxyl-terminal region) or by covalent derivatives of the noncompetitive blockers whose multisubunit high-affinity site is expected to lie in or near the ion channel.

This still hypothetical scheme for the transmembrane folding of the receptor polypeptides shows large correspondences with the organization of the chromosomic gene (*132*). The human chromosomic gene encoding the α subunit precursor is split by eight noncoding sequences, or introns, into nine coding exons referred to as P1 to P9 (Fig. 4C). The introns, which altogether amount to approximately 15 kilobases, do not intervene randomly in the coding sequence of 1383 base pairs. P1 encodes most, but not all, of the signal peptide; P6 encodes helix I plus a fragment of the large hydrophilic domain; P7 encodes helices II and III; and P9 encodes helix IV. The large extracellular domain is coded by exons P3, P4, and P5 (plus part of P2 and P6), and the small cytoplasmic domain is coded by P8. On the other hand, the hypothetical amphipathic helix A is split by an intron. These correlations may have evolutionary implications; for instance, some of these domains might be shared by the genes coding for different receptors or ion channels. In addition, they further support the view that the acetylcholine binding site and the ion channel are carried by distinct segments of the chain and thus that their interaction is indirect or allosteric (*86*).

The Receptor in Its Membrane Environment

In the adult electromotor or neuromuscular junction, the distribution of the acetylcholine receptor is largely restricted to the cytoplasmic membrane which underlies the nerve ending. In this region, the receptor density reaches a value of about 15,000 to 20,000 molecules per square micrometer (*184*), with a lipid-to-protein ratio in *Torpedo* strongly in favor of the proteins (0.4 to 0.5) (*185, 186*). The lipid composition of this membrane is characterized by a high content of unesterified cholesterol and the predominance of long-chain highly unsaturated fatty acids that are precisely those with which the purified receptor reacts preferentially in pure lipid monolayer experiments (*185*).

Functional reconstitution of detergent-purified receptor into lipid vesicles (*27*) and planar bilayers (*28*) reveals a strong dependence on the presence of lipids throughout the isolation and purification procedures. These lipids, such as asolectin, have been found to prevent the receptor from shifting to a "degenerate" low-affinity state for agonists where they no longer display the characteristic allosteric transitions (*187*). Pure phospholipids are inefficient, but neutral lipids such as tocopherol (*188*) and cholesterol (*189–191*) exert a strong protection or even enhance the recovery of acetylcholine-dependent ^{22}Na flux. During receptor purification, up to 60 molecules of phospholipids still bind (some covalently) per receptor molecule (*192*), a number in agreement with that corresponding to the immobilized component observed by ESR spectroscopy in the boundary of the receptor in native membrane fragments (*192*) and reconstituted vesicles (*193*).

Fatty acids added to the membrane (*194*) or generated by phospholipase A_2 action (*195*), detergents (*194*), or local (*101*) and general (*170, 171*) anesthetics strongly block the permeability response to agonists and modify the rapid binding kinetics, possibly via allosteric effects mediated by (i) the multiple, low-affinity histrionicotoxin-insensitive sites for noncompetitive blockers (*104*) that are possibly located at the boundary between the transmembrane α helices and the lipid bilayer, or (ii) a direct binding to the high-affinity site. Reciprocal effects of agonist binding on the relations of the receptor with the neighboring lipid phase are suggested by the fluorescence changes of hydrophobic probes, which covalently react with the receptor via the lipid bilayer (*196*), or by studies on the partition of a hydrophobic cation (*197*). The lipid environment of the receptor thus exerts a critical, although mostly permissive, action on the regulatory properties of the receptor.

The contribution of the protein environment might be critical as well. On the cleft side, components of the basal lamina may interact with the receptor and contribute to its clustering during synapse formation (*198*). On the cytoplasmic side, electron microscopy reveals "condensations" beneath the receptor layer (*199, 200*) and morphological relations with the cytoskeleton (*201, 202*). Proteins with an apparent molecular weight of 43K on SDS gels purify together with the receptor-rich membranes (*203*) and give on two-dimensional gels one major component, referred to as the 43K protein, and at least two others identified respectively as cytosolic creatine phosphokinase (*204, 205*) and cytoplasmic actin (*205, 206*). Selective proteolysis (*50*), labeling with isotopic iodine (*61*), and gold labeling with specific monoclonal antibodies (*207, 208*) have shown that the 43K protein exclusively faces the cytoplasmic side of the membrane and distributes along with the acetylcholine receptor. Brief exposure of the receptor-rich membranes to *p*H 11 (*209*) or lithiumdiiodo salicylate (*206, 210*) releases the 43K and other peripheral proteins without significant changes of the receptor functional properties monitored by ion flux measurements of rapid binding of agonists and noncompetitive blockers (*187, 209, 210*).

In contrast, elimination of the 43K protein destabilizes the receptor to heat treatment (*211*) or proteolytic attack (*51*) and enhances its motion as monitored with a spin-labeled (*212*) or phosphorescent (*213*) derivative of α-bungarotoxin and by electron microscopy (*214*). Its binding to the cytoplasmic domain of the receptor, at least by way of the β subunit (*215*), thus strongly immobilizes the molecule. A contribution of the heavy-form dimer to this process has been suggested (*15*). The close relation between interme-

diate filaments and the 43K protein suggests that this highly insoluble, cysteine-rich molecule may serve as an "intermediate piece" between the cytoskeleton and the postsynaptic membrane (*15, 202*) and thus contribute to the highly local distribution of the receptor protein.

Perspectives of Receptor Research: Toward a Molecular Biology of Learning

The molecular organization of the acetylcholine receptor conciliates features that reflect its function as an allosteric protein and its transmembrane disposition associated with its role in intercellular communication. As an allosteric protein (*86, 87*) it shows homotropic cooperative interactions between topographically distinct acetylcholine sites and heterotropic interactions between the same sites, the ion channel, and various allosteric sites for noncompetitive blockers. Conformational transitions between discrete states mediate these interactions; the pharmacologically active ligands, agonists, competitive antagonists, and noncompetitive blockers act through the differential and selective stabilization of some of these preexisting states. Despite a "baroque" $\alpha_2\beta\gamma\delta$ quaternary structure, the homology between subunits may serve as the structural basis of a cryptic rotational symmetry. An orientation of this axis perpendicular to the plane of the membrane is compatible with a transmembrane polarity of the tertiary folding of the subunits, giving access to the neurotransmitter on the cleft side and anchoring to the cytoskeleton at a well-defined locus on the cytoplasmic side, and yielding an ion channel spanning the membrane. Future work should lead to the description of the three-dimensional organization of the molecule and of its conformational transitions at the atomic scale.

Because of their role in intercellular communication, postsynaptic receptors might constitute critical targets for activity-dependent, long-lasting changes of synapse efficacy (*15, 216*) in addition to, or as alternatives of, presynaptic modifications (*217*). The still hypothetical extension of our present knowledge about the acetylcholine receptor to central receptors leads to the suggestion of a number of plausible but entirely speculative models for short- and long-term learning at the neuronal level. A first attractive hypothesis (*216*) is that desensitization may contribute to short-term regulation in the second-minute time scale of synapse efficiency by modulating its own state of activity [see (*159*)]. The same slow transition between a resting, activatable, and desensitized-refractory state might be affected by "allosteric" signals generated by synapses impinging on the same neuron, thus mediating a selective association between these synapses as a function of their state of activity (*216*). In the case of the nicotinic receptor, examples of such "endogenous" allosteric effectors are (on the outside surface) still unknown noncompetitive blockers that may include opiates (*218*) or substance P [in chromaffin cells (*219*)] and (on the cytoplasmic side) Ca^{2+} ions (*89, 164, 220*) and of course the membrane electrical potential (*154*). Models of heterosynaptic regulation resulting in functional association between distinct pathways, including, in particular, the Hebb synapse, have been formalized on this basis (*216*). These reasonings have been extended to eventual mechanisms of "resonance" between spontaneous and externally evoked activity as a basis for selection of neuronal assemblies (*221*). As allosteric proteins, neuronal receptors would then serve as molecular devices specialized in space and time "integration" of synaptic and neuronal signals.

Such short-term changes of synapse efficacy, in the minute time scale, might hypothetically be made to last longer by covalent modification of the receptor protein (and of the 43K protein), such as phosphorylation (*47, 222, 223*), methylation (*224*), and glycosylation (*130, 131*). Other possibilities are eventual activity-dependent changes in local lipid environment or differential association with peripheral proteins from the basal lamina or the cytoskeleton (such as the 43K protein).

In addition to regulation of ion channel properties, critical steps in the biogenesis of the receptor (*122, 123*) in its incorporation (*125*) and metabolic stabilization (*15, 225*) in the postsynaptic membrane might become critical targets for long-lasting traces of synaptic activity. Motor innervation is already known to regulate the biogenesis of muscle extrasynaptic acetylcholine receptor (*225*) at least at the level of transcription (*226*), and electrical activity plays a critical role in this regulation by intracellular Ca^{2+} (*227*) or cyclic nucleotides or both (*228*). Long-term changes in the degradation rate (and channel mean open time) of the synaptic acetylcholine receptor also occur in the course of the maturation of the neuromuscular junction, but their activity dependence remains uncertain. Work on the acetylcholine receptor thus offers hypotheses on plausible molecular mechanisms of learning which remain to be tested with specific nerve cells under unambiguous physiological learning conditions.

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