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Nicotinic Acetylcholine Receptor Superfamily of Ligand-Gated Ion Channels

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Ligand-gated ion channels provide for the rapid dialogue between cells of the central nervous system, converting a chemical neurotransmitter signal released from one cell into an electrical signal that propagates along the target cell membrane. Now the action of psychotropic drugs links particular receptor subunits with behavior and disease states. As knowledge of three-dimensional structures increases, designed neuropharmaceuticals can emerge through structure-based drug design. At the biochemical level, analysis of the nicotinic acetylcholine receptor (AChR) has been practically the only paradigm for this type of receptor. But this situation is changing as molecular cloning reveals a plethora of neurotransmitter receptors and hints at how these molecules are localized and diversify during development. As information accumulates about other receptors, the acetylcholine receptor still serves as a benchmark for comparison.

To date, five families of ligand-gated receptors that mediate information transfer in the brain and neuromuscular junction have been characterized at the level of their sequences. They are the excitatory nicotinic AChRs of neuromuscular and neuronal origins which conduct cations, excitatory neuronal kainate type glutamate-activated channels which conduct cations, inhibitory neuronal GABA and glycine-activated channels which conduct chloride, and the the sarcoplasmic reticulum ryanodine receptor which conducts calcium. Different in size and sequence, these families of channels comprise a superfamily whose subunits share several features: four putative membrane-spanning regions denoted M1–M4; strong sequence homology in these hydrophobic transmembrane sequences, especially in M2, the hydrophobic stretch of ~20 amino acids that probably lines the ion-conducting channel; and probable quasi-symmetric pentameric arrangement of similar or identical subunits. A homologous cystine-bridged loop is found on the extracellular side of subunits. Diversity within a family is provided by expression of genes that substitute for one or other of the subunits that together form functional receptors. We analyze findings that bear on how these ligand-gated ion channels function and illustrate how in the absence of high-resolution structures much has been deduced about how their structures support function.

Nicotinic AChR Family

Nicotinic AChRs conduct $\sim 10^4$ sodium ions/ms in response to binding of cholinergic agonists. Experiments using photoactivated ligands to produce agonist concentration jumps show that channel opening follows less than 10 μs after ligand binding (Krouse et al., 1980). The flow of sodium ions into the cell rapidly short-circuits the normal ionic gradient maintained across the plasma membrane by ATP driven pumps, and so depolarizes it. The typically ca. −50-mV potential difference across the membrane is reduced to ca. −5 mV, thus transducing a chemical signal into an electrical one that signals muscle contraction at the neuromuscular junction. In the central nervous system, AChR subunits encoded by different genes are responsible for excitatory transmission. Individual channel openings are brief, averaging 1 ms for junctional receptors at a holding potential of −100 mV in the frog neuromuscular junction (Katz & Miledi, 1971; Magleby & Stevens, 1972). If agonist is present continuously, the AChR fluctuates rapidly between conducting and nonconducting states for the next several hundred milliseconds before entering into a nonconductive, no longer responsive desensitized state. There are at least two kinetically differentiable forms of desensitization, a fast-onset "intermediate" form that occurs in the millisecond time scale observed by rapid mixing techniques by Hess et al. (1979) and by patch-clamp methods by Sakmann et al. (1980) and a slow-onset equilibrium form (Adams, 1981). Desensitized AChR binds agonist about

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1000-fold more tightly than does the initial, resting state conformation from where the channel is opened, though the channel remains closed (Katz & Thesleff, 1957; Weber et al., 1975). The rapidly desensitized form binds about 20-fold more tightly than required for opening [Cash & Hess, 1980; reviewed by Ochoa et al. (1989)]. In vivo, the enzyme AChesterase, located on the basal lamina between neuron and target cells, rapidly hydrolyzes ACh, thereby halting its effect before desensitization could occur; thus, the physiological role of desensitization is unclear.

Electrocytes which compose the electric organ of the electric rays *Torpedo* and *Narcine* and electric eel *Electrophorus* contain close-packed AChR in postsynaptic regions of the plasma membrane. These differentiated cells develop from muscle cells in the embryonic fish (Aronstam, 1982), and their AChR are highly homologous in sequence and properties to those from mammalian neuromuscular junctions, as demonstrated by both their immunological cross-reactivity (Lindstrom et al., 1978) and their primary sequences (Conti-Tronconi & Raftery, 1982). Tightly binding ligands, such as α-bungarotoxin from the venom of the snake *Bungarus multicinctus* ($K_d \sim 10^{-11}$ M), greatly facilitated initial purification of AChR. Because AChR can be isolated in large quantity from electric organ, it has yielded most of the biochemical insight into the AChR family, different aspects of which have been reviewed by Lindstrom et al. (1987), Guy and Hucho (1987), McCarthy et al. (1986), Merlie and Smith (1986), Stroud and Finer-Moore (1985), and Popot and Changeux (1984).

A Pentamer of Homologous Subunits Surrounds the Channel

The AChR is a complex of five homologous transmembrane glycoprotein subunits that surround a central ion channel (Kistler et al., 1982). In *Torpedo* there are four gene products designated α, β, γ, and δ of molecular masses 52.4, 56.2, 63.2, and 65.9 kDa, variously 0–30% higher than their apparent molecular masses on SDS–PAGE (40, 50, 60, and 65 kDa). From the purified α subunit, the first 20 residues of *Torpedo marmorata* AChR (Devillers-Thiery et al., 1979) were sequenced. Subsequently, the first 56 amino-terminal residues of all four subunits of *Torpedo californica* AChR were similarly defined, showing that they were all closely homologous (35–50%) and therefore evolutionary related proteins (Raftery et al., 1980). By quantitative sequence analysis Raftery et al. (1980) demonstrated that the AChR is a pentamer, with a stoichiometry of $\alpha_2\beta\gamma\delta$, in agreement with the conclusions of Reynolds and Karlin (1978) and Lindstrom et al. (1979) on the basis of analysis of molecular weight. Further amino acid analyses from *Electrophorous* (Conti-Tronconi et al., 1982a) embryonic mammalian muscle (Conti-Tronconi et al., 1982b) and avian muscle and neuronal tissue (Conti-Tronconi et al., 1985) first established the existence of a family of AChRs. On the basis of the *T. californica* or *T. marmorata* sequences, molecular cloning soon gave complete sequences for the AChR chains. In 1982 four groups reported cloning and partial or complete sequencing of cDNAs of individual AChR subunits from either *T. californica* or *T. marmorata* (Sumikawa et al., 1982; Ballivet et al., 1982; Giraudat et al., 1982; Noda et al., 1982). After alignment, sequences of α (Sumikawa et al., 1982; Noda et al., 1982; Devillers-Thiery, 1983, β and δ (Noda et al., 1983a), and γ subunits (Claudio et al., 1983; Noda et al., 1983b) show 19% identity and 54% homology (Noda et al., 1983b). mRNAs transiently expressed on the surface of *Xenopus* oocytes show that α subunits alone bind α-bungarotoxin but that the coexpression of either the γ or δ subunit is required for specific agonist binding (Kurosaki et al., 1987). Stable expression of all four subunits has been achieved in fibroblast L cells (Claudio et al., 1987). Expressed AChR lacking either the β, γ, or δ subunit exhibits limited ion conduction in response to agonist binding as measured by whole-cell voltage clamp. No flux response is observed when only individual subunits are expressed (Kurosaki et al., 1987), though this is not generally true for neuronal AChR or for other superfamily members where homooligomeric arrangements are fully functional.

AChR from *Torpedo* forms ordered two-dimensional arrays (Ross et al., 1977; Stroud, 1983) and ordered tubular vesicles with crystalline lattices. These were used for two-dimensional image filtration (Kistler & Stroud, 1981) and combined with X-ray diffraction to give a composite three-dimensional reconstruction (Kistler et al., 1982). AChR in these tubular membrane vesicles are composed largely of dimeric AChR (Kistler et al., 1981) and crystallize with dimeric symmetry (Brisson & Unwin, 1984; Mitra et al., 1989) which is independent of the disulfide bonds that cross-link 80% of AChR (Mitra et al., 1989) by cross-linking between C-termini of the δ chains of adjacent molecules (DiPaola et al., 1989a). These crystals gave rise to three-dimensional reconstructions from tilted samples at 25 Å both in stain and in ice (Brisson & Unwin, 1985), from helical diffraction (Toyoshima & Unwin, 1988), and by combined tilting stage electron microscopy at 22 Å and X-ray diffraction to 12.5-Å resolution perpendicular to the membrane plane (Mitra et al., 1989) (Figure 1). The quasi 5-fold symmetry of the AChR is seen clearly on the synaptic side and in projected views (Brisson & Unwin, 1985; Mitra et al., 1989). Projections onto the membrane plane show the 5-fold symmetry most clearly after removal of the 43-kDa peripheral proteins as in Figure 2 or after extensive proteolysis (i.e., if protease inhibitors are left out) since the subunits are up to 30% different in mass.

Two Binding Sites of Different Affinity Optimize Response

The two α chains, most associated with ligand binding at least for toxin molecules, lie in nonequivalent environments. They are separated by an angle of 144° ± 4°, measured most accurately from the angle between pairs of anti-α chain monoclonal Fab fragments on noncrystalline AChR by immunoelectron microscopy. This is precisely two sectors of a pentagon, around the axis (Fairclough et al., 1983); they are not adjacent in the pentamer, also deduced by electron microscopy of Zingsheim et al. (1982), and Karlin et al. (1983). Binding of (at least) two ACh molecules is required to effect channel opening since the Hill coefficient for response is n = 2.0 (1.97 ± 0.06) (Neubig & Cohen, 1980). There is no cooperativity in binding ACh; simply two molecules of ligand must bind noncooperatively to open the channel. In addition, the sites one presumably associated with each of the two α chains have different affinities, a requirement that can be rationalized in both energetic and structural terms. Following the arrival of an action potential at the presynaptic terminal, ACh concentration within the synaptic cleft increases from $\sim 10^{-8}$ M to $\sim 0.5\ 10^{-3}$ M (Katz & Miledi, 1977; Kuffler & Yoshikami, 1975). These values place constraints on the equilibrium constants for ACh binding to closed and open AChR and correspondingly on the energy derivable from binding that can be used for opening the channel. Assessment of the energy that can be derived from one-site binding alone shows that two are required to effect opening and that two different affinities for ACh permit optimum activation and termination times (Jackson, 1989). The system is beautifully

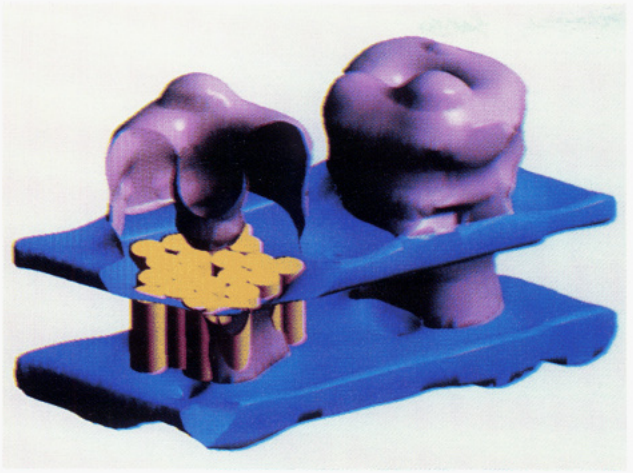

FIGURE 1: Outer surface of the AChR structure seen from a three-dimensional electron microscopic and X-ray diffraction structure analysis to 22 Å × 12.5 Å resolution (Mitra et al., 1989). The surfaces enclose positive densities. The density for the bilayer, in blue, appears as horizontal planes of densities which correspond to the dense phosphatidyl head groups that are 41-Å apart (Stroud & Agard, 1979). The bundles of cylinders, shown to scale, have the dimensions of 30 Å long, close-packed α helices in the hydrophobic center of the bilayer-spanning region, as suggested by X-ray diffraction and sequence analysis, equivalent to ~20 amino acid residues in length, and are shown in a cutaway for one of the AChR molecules in the dimer. Relative to the entry well the closest helices are most likely M2, left-handed supertwisted, on the basis of model building. The synaptic side of these helices forms the base of the well. The 25 Å diameter infundibulum extends from the outermost extremity to just below the surface level of the phospholipid bilayer.

matched to optimize the speed and veracity of signaling.

The arrangement of other subunits is less well established, analyzed by less direct methods than for the α chains. By immunoelectron microscopy of labeled molecules and chemical cross-linking, the order $\alpha\beta\alpha\gamma\delta$ seen from the synaptic side was deduced for *T. californica* (Kistler et al., 1982; Fairclough et al., 1983) and confirmed by electron microscopy for *T. marmorata* (Kubalek et al., 1987). It was also the most favored model from cross-linking of Hamilton et al. (1985). However, expression of only α and γ or of α and δ chains together in fibroblast cells shows that carbamylcholine, a stable agonist of ACh, has different affinities for each pair, as they are different for the two sites on AChR. Curare also seems to implicate two nonequivalent binding sites (Pedersen & Cohen, 1990). When coexpressed, α and β chains do not associate effectively and so do not bind ligand (Blount & Merlie, 1989). One interpretation of these results is that the ligand binding sites lie in α–γ and α–δ interfaces, and these would not be quasi-equivalent with respect to α in the $\alpha\beta\alpha\gamma\delta$ arrangement. Others have evidence that there are more than two activating sites at similar sites on the pentameric structure (Dunn & Raftery, 1982; Dunn et al., 1983; Conti-Tronconi & Raftery, 1986), and this becomes especially relevant in considering other superfamily members where homooligomeric channels, with as many as five equivalent interfaces are formed. In locust, homooligomers of an α-like subunit form ion channels (Breer et al., 1985). Thus some further confirmation of subunit arrangements in AChR beyond the α chains and their roles is needed.

Conductance and Electrostatics of the Vestibule

The extracellular domain of the AChR forms a cylindrical vestibule, which extends 54 Å above the membrane plane (Figure 1). Individual subunit heights above the bilayer surface range from 50 to 60 Å, corresponding to the ~30% difference in subunit mass there. The outer diameter ranges from 74 to 81 Å, and the solvent-accessible surface area of the entire extracellular region of the structure is 18 500 Å^2, of which about 20%, or 3780 Å^2, forms the lining of the vestibule. A protein wall 24.5 ± 1.5 Å thick surrounds the entry well, insulating the channel from the effects of changes in membrane surface charge. This thickness is typical for the dimensions of an antiparallel β-barrel structure predicted for the extracellular domain of AChR from amphipathic secondary structure analysis (Finer-Moore & Stroud, 1984; Finer-Moore et al., 1990) (Figure 3).

High channel conductance for cations may be due in part to a negatively charged vestibule which would serve to concentrate positively charged ions. The calculated p*I* is 4.77 for the predicted extracellular region, from aligned sequences[1] 1–228 of the five subunits that contain 150 Glu and Asp, 98 Lys and Arg, and 30 His, not atypical for soluble proteins. Thus the net charge on the extracellular domain at pH 7.0 is calculated to be −50, and the average charge density is ~1 charge per 67 Å^2 of total solvent-accessible surface. Assuming an even distribution, the expected net charge within the vestibule would be an excess of 10 negative charges, comprised of 31 negative and 21 positive groups. The outside of the complex which contacts the negatively charged lipid head groups may carry somewhat more positive charge, while the inside surface that forms the entry well could be more negative. The excess negative charge density can contribute to the channel's cation selectively by repelling anions and concentrating cations in the channel mouth. Dani (1986, 1989) and Dani and Eisenman (1987) show quantitatively how negative surface charge within a vestibule of about these dimensions and a pore of low charge density containing a short, narrow region which holds about six water molecules can generate many of the observed electrophysiological properties of AChR. Significant in this regard, the GABA and glycine receptors which conduct anions have excess positive charge of about the same amount in their vestibular region, suggesting that charged vestibules may be a general mechanism of enhancing anion or cation selectivity.

Just below the level of the membrane surface leading to the central pore, the diameter of the entry well narrows from 25 to ~7–8 Å on entry to the transbilayer region; the diameter is estimated from overlap of many AChR images limited by the resolution of the microscope (Fairclough et al., 1983); it is not resolved in reconstructions from crystalline samples at resolutions of ~20 Å, which exceed this dimension and therefore only establish that it is less than about 15 Å (Toyoshima & Unwin, 1988; Mitra et al., 1989). X-ray scattering utilizing five-wavelength anomalous dispersion shows that there are discrete cation binding sites located within the transbilayer region in the resting state of AChR (Fairclough et al., 1986). Thus, the central pore is almost certainly the gated ion conducting channel, probably surrounded by a twisted bundle of transmembrane α-helices as schematized in Figure 4. If we preserve the quasi 5-fold symmetry, optimum side-chain packing is achieved with a ~12° tilt with respect to the axis and a left-handed twist around the ~7.7-Å pore in the center, which makes the angle between helix axes ~20°. Constraints on the inner helix packing, at the polar/nonpolar interface, are probably more stringent than for the external helices, as suggested by the open structure of the hydrophobic helices A and B in the photoreaction-center crystal structure which contact the lipid bilayer (Deisenhofer et al., 1985).

[1] Sequence numbers in the text refer to a sequence alignment as by Stroud and Finer-Moore (1985).

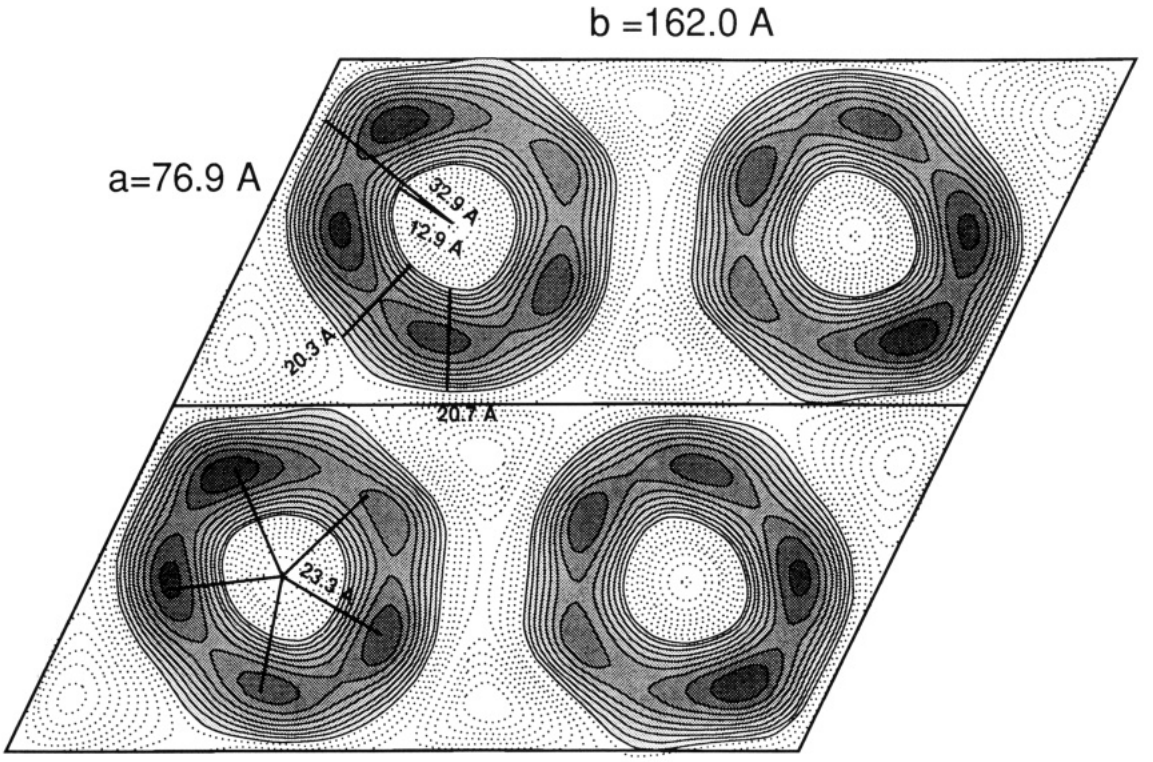

FIGURE 2: Filtered projection images of alkali-stripped AChR. The pentameric nature of the structure is most apparent after removal of peripheral proteins. The subunit arrangement $\alpha\beta\alpha\gamma\delta$ viewed from the synaptic side of AChR was deduced by Kistler et al. (1982). Fairclough et al (1983), and Unwin et al. (1988). Solid lines enclose the stain-excluding protein region. Several dimensions of the projected view are shown. The unit-cell dimensions for the alkali-stripped form are a = 76.9 ± 2.0 Å, b = 162.0 ± 3.2 Å, and included angle γ = 116.0° ± 1.8°.

Noncompetitive Channel Blockers Implicate M2 in Lining the Channel

Noncompetitive channel blocking compounds act by blocking the open ion channel, often in an agonist- or voltage-dependent fashion. Therefore, a binding site for noncompetitive blockers delineates portions of the AChR which form the ion channel. There is one high-affinity binding site for noncompetitive blockers per AChR (Heidmann et al., 1983). Initially, photolabeling of AChR both in the resting and equilibrium-desensitized state with the noncompetitive blockers trimethisoquin, phencyclidine, histrionicotoxin, and chlorpromazine (CPZ) was studied (Oswald & Changeux, 1981). Addition of agonist stimulated incorporation of trimethisoquin, phencyclidine, and histrionicotoxin into the δ subunit, while incorporation of CPZ into all four subunits was enhanced. Triphenylmethylphosphonium (TPMP) photolabeled the α subunit of resting-state AChR but showed agonist-dependent labeling into the δ and to a lesser extent the β subunit (Muhn & Hucho, 1983). In long-term labeling experiments, quinacrine mustard (Kaldany & Karlin, 1983) and meproadifen mustard (Dreyer et al., 1986) were shown to alkylate the α and β subunits selectively in response to agonist.

In studies of the kinetics of photolabeling in the millisecond time range using stopped-flow methods, simultaneous addition of agonist and CPZ caused a rapid incrase (100-ms time scale) in photolabeling of all four subunits of the AChR (Heidmann & Changeux, 1984). Photolabeling by TPMP on the δ and β subunits (Muhn et al., 1984) and by quinacrine azide on the α and β subunits (Cox et al., 1985) was observed, with similar rate constants. Thus, the noncompetitive blockers bind to the resting and equilibrium-desensitized states of the AChR, as observed for nonreactive noncompetitive blockers such as phencyclidine (Oswald et al., 1983; Karpen & Hess, 1986). These results are all consistent with an agonist-induced change in the configuration of the binding site within the channel upon activation of the channel.

The site of CPZ incorporation into the δ subunit of AChR in the equilibrium-desensitized state was found to be serine 266 (see Figures 4 and 5) in M2 (Giraudat et al., 1986). TPMP is also incorporated, again under desensitizing conditions, into the same δ-subunit serine (Oberthur et al., 1986). Subsequently, homologous M2 serine 266 was found to incorporate TPMP in α and β subunits (Hucho et al., 1986) and along with a nearby leucine to incorporate CPZ in the β (Giraudat et al., 1987) and α subunits (Giraudat et al., 1989). These and other considerations led Hucho et al., (1986) and Giraudat et al. (1987) independently to present a structural model wherein the M2 helices of the AChR subunits pack to form the lining of the AChR ion channel, with a noncompetitive (open channel) blocker binding site located in the region of the homologous serines 266 (Figures 4 and 5). Mutagenesis strongly suggests that it is correct.

An additional noncompetitive blocker binding site, on the putative transmembrane helix M1, is found for quinacrine azide (Karlin et al., 1986) which is competitive with CPZ (DiPaola et al., 1990b), though M1 is not labeled by CPZ (Giraudat et al., 1989). This is consistent with M1 forming part of the channel walls, perhaps around M2. M1 contains a conserved pair of amino acids -Pro238-Cys239- near the center of M1 that may have a role in channel conductance. On the basis of labeling with a fluorescent maleimide, Cys239 lies as a free sulfhydryl within a hydrophobic pocket (Marquez et al., 1988).

M2 Then M1 and M3 Are Most Critical to Ion Conductance

Transient expression of AChR subunits in *Xenopus* oocytes allows the study of AChR subunits altered by directed mu-

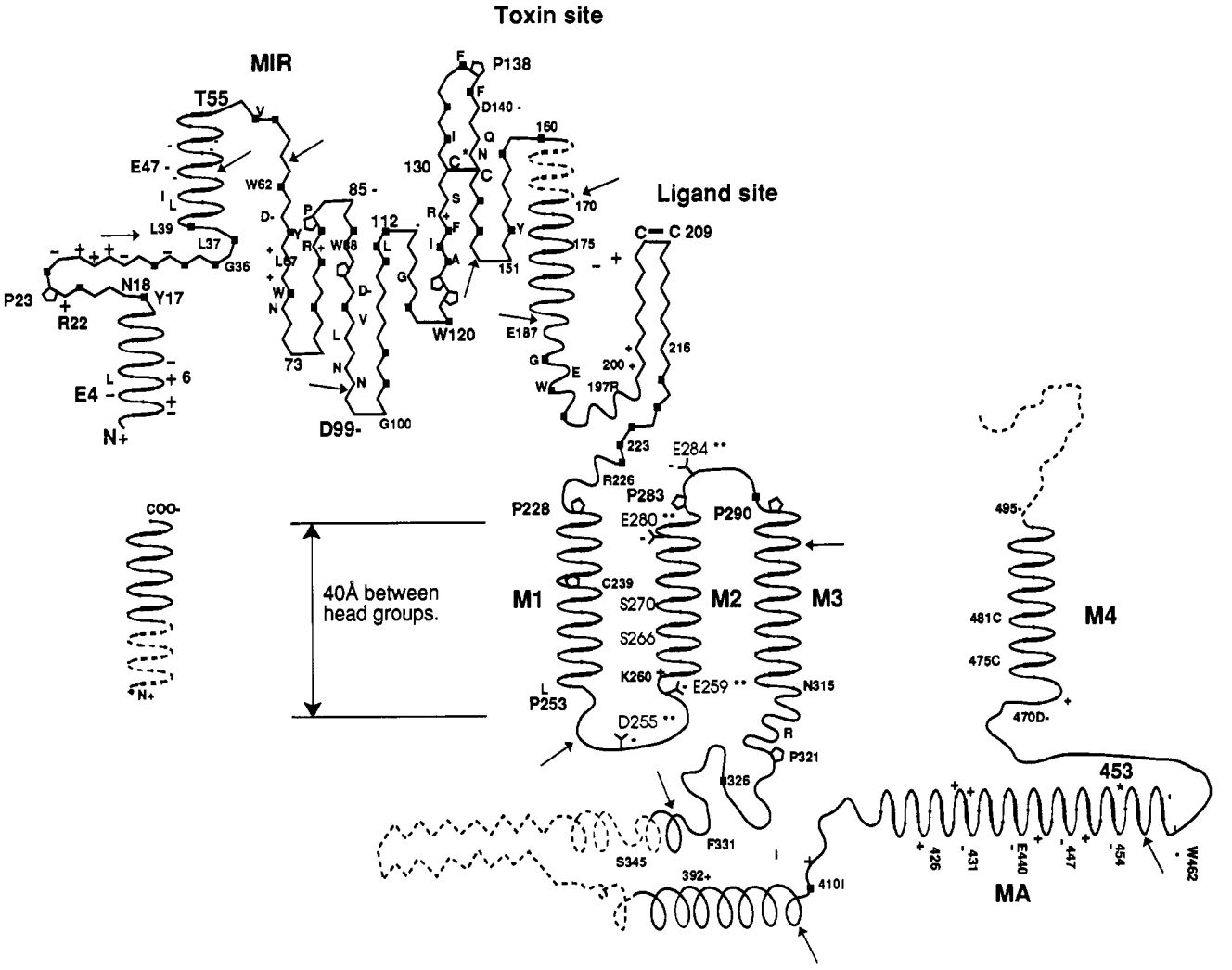

FIGURE 3: Consensus model of the topology of a neuromuscular AChR subunit and secondary structure predicted by amphipathic analysis (Finer-Moore & Stroud, 1984; Stroud & Finer-Moore, 1985). The four hydrophobic, putative membrane-spanning α helices are labeled M1–M4. Residues in M2 whose mutation causes change in conductance are indicated in narrow letters with (**) on those that are charged. The conserved cysteines, known to form a disulfide-linked loop in all four neuromuscular AChR subunits C130–C144, enclose the conserved site of N-linked glycosylation (*), found in all neuromuscular AChR subunits and GABA receptor β subunits. The adjacent, disulfide-linked cysteines at the agonist binding site, found only in AChR α subunits, are C208 and C209. Square blocks indicate conservation of hydrophobic residues in the extracellular side that would tend to fold more toward the inside. Letters identify the more usual residue of highly (not necessarily totally) conserved residues. Positive and negative signs identify regions that generally carry charged side chains. Conserved prolines often occur at the ends of transmembrane helices. Dashed lines indicate variable-length substitution between species. Arrows indicate positions of common exon/intron boundaries.

tagenesis. Altered *T. californica* α subunit, coexpressed with wild-type β, γ, and δ subunits, showed that deletion of all or part of the hydrophobic, putative membrane-spanning regions M1, M2, M3, and M4 (Figure 3) leads to greatly reduced α-bungarotoxin binding and loss of acetylcholine-gated ion flux (Mishina et al., 1985). The involvement of M2 in ion channel formation is indicated by mutations in M2. Imoto et al. (1988) show that mutations in charged residues at the ends of M2 affect channel conductance, while Leonard et al. (1988) show that the duration of block by the pure open channel blocker QX-222, which only binds to AChR in the open state, decreased monotonically with the number of serine residues removed from the polar face of M2. In all four subunit types, there are positively and negatively charged amino acids at both ends of M2 (Figure 5). Symmetrical packing of M2 from each subunit to form the lining of the ion channel would place these charged residues in rings at each end (Figure 4). Replacement of glutamate, aspartate, or glutamine by glutamine or lysine at positions 280 on the extracellular entry side and 255 and 259 on the cytoplasmic side reduced the directional flow of cations especially from that side, producing rectification. The directional decrease in conductance was almost linearly dependent on the decrease in net negative charge on the entry side (Imoto et al., 1988). This supports a model in which rings of negative charge at each end of M2 attract cations to the channel entrance from that side. Changes at position 259 produce a larger alteration in the conductance than do changes at either 255 or 280 but do not produce rectification. It is as if 259, near the cytoplasmic surface, lies at the narrowest portion of the helix bundle. This is consistent with measurements of streaming potentials which show that the narrowest region is short (Dani, 1989) and with the location of covalent attachment sites of channel blocking compounds CPZ (Giraudat et al., 1986) and TPMP (Oberthur et al., 1986) at serine 266 near the cytoplasmic side of M2. Replacement of glutamate or aspartate by lysine, or conversely lysine or arginine by glutamate in other portions of the AChR including MA, had no effect on channel conductance but sometimes impaired expression of AChR. Replacement of serines 266 and 270 by alanine in M2 of mouse α, β, and δ subunits also led to irregular rectification and much diminished outward current (Leonard et al., 1988), suggesting that these serine residues near the cytoplasmic end of the putatuve M2 may lie at the narrowest region of the open channel (Figure 6).

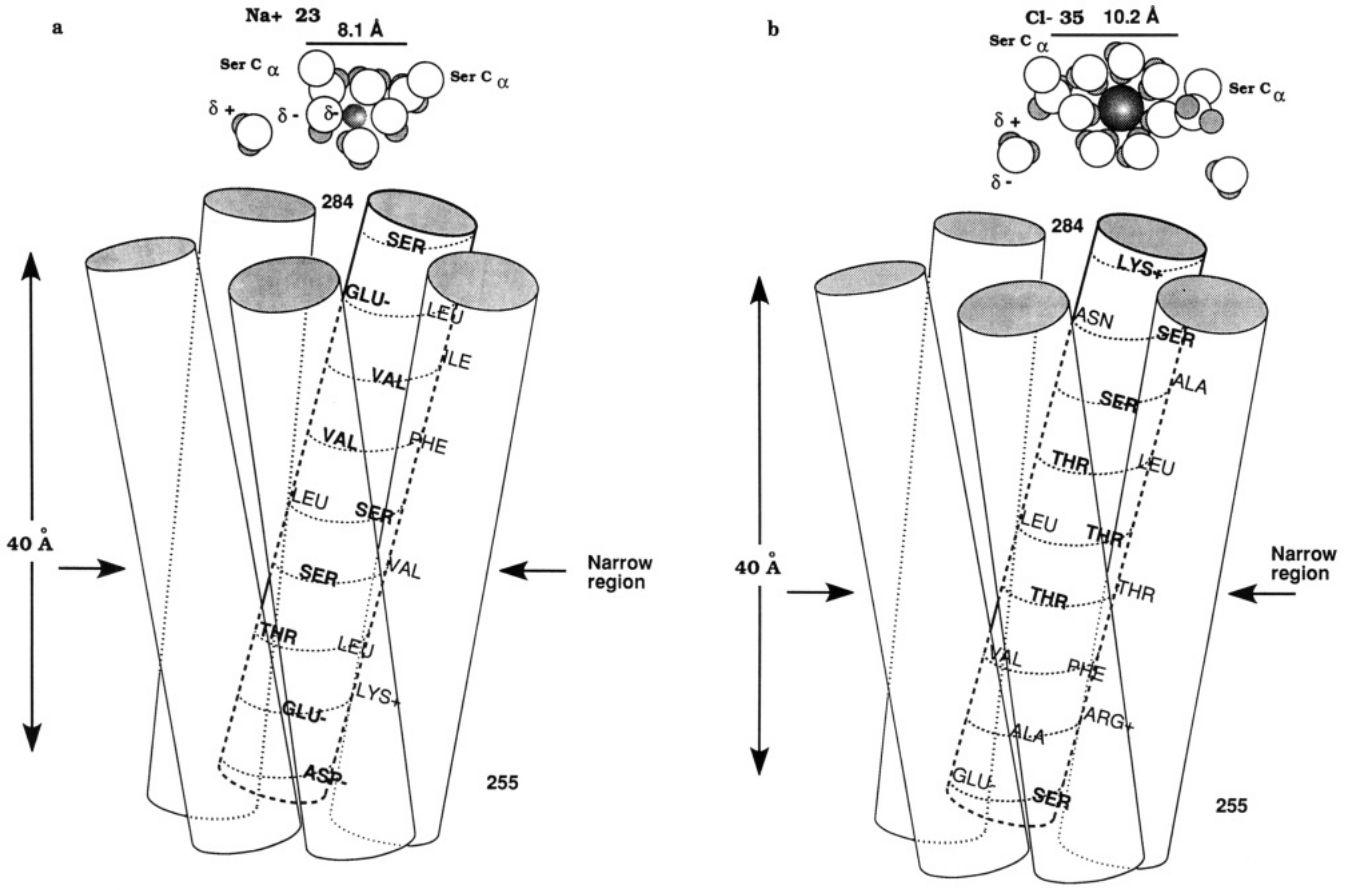

FIGURE 4: Cut-away depiction of the approximate size and sequence of M2 α helices packed around a central channel, representing the open state of the channel: (a) in cation-conducting nicotinic AChR receptor, the α-chain sequence is displayed in precise α-helical configuration. (b) In an anion-conducting channel, GABA α chain is depicted. Reduction of negative charge in (a) at 280 decreased inward current (downward); at 255 it reduced outward current, suggesting that these residues may concentrate ions for passage. Changes at 259 had the largest effect on conductance, suggesting that the channel is narrowest, near 259 (Figure 5). The distances are approximately to scale, as they are in the sketch of hydrated sodium (a) and chloride ions (b). Around the ions the inferred different orientations of water molecules and serine side chains are indicated. Hydroxyl groups from serines or threonines within M2 have potential for back-bonding between serine hydroxyl and the carbonyl groups of residues four residues earlier in the sequence, which will present the lone pair of electrons, the $\delta-$ on the oxygen toward the cation channel. The chloride ion is significantly larger, has a smaller energy of hydration, and probably associated with protons on the hydroxyls. The chloride channel sequences are richer in hydroxyl side chains and threonine rather than serine.

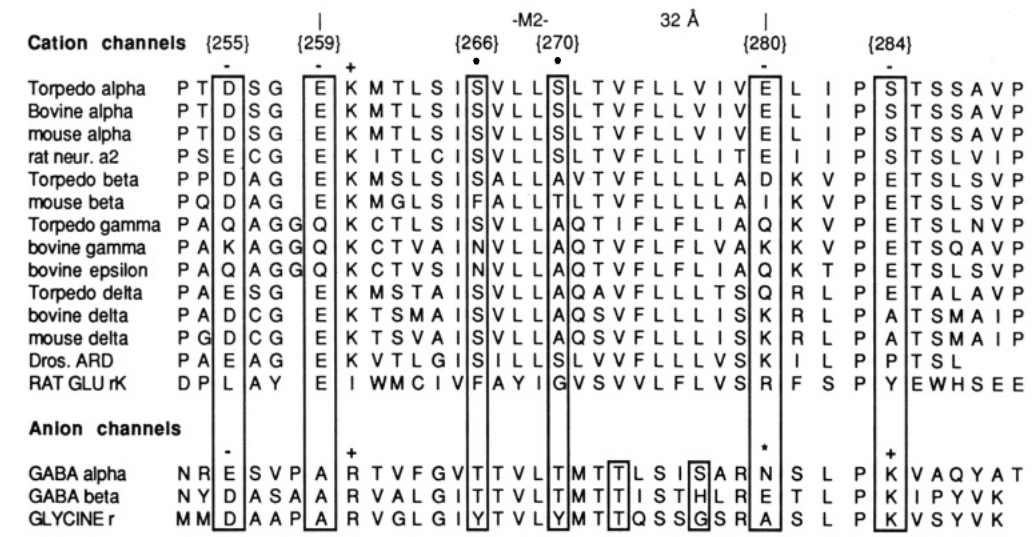

FIGURE 5: Sequences of M2 regions in cation-conducting, ACh, and glutamate-activated channels and in anion-conducting, GABA, and glycine-activated channels illustrate the higher number of polar residues in the anion-conducting channels. Mutation of residues at consensus sequence numbers 255, 259, 266, 280, and 284 affects conductance (Imoto et al., 1988), as do serines at 266 and 270 (Leonard et al., 1988), all of which are expected to lie on the same α-helical face. The polar groups at 259 have the greatest effect on conductance, suggesting that this is the narrowest region within the channel. Rings of charged residues on either side seem to determine conductance from that side.

When the transmembrane helices were replaced by foreign sequences from vesicular stomatitis virus glycoprotein (GP) or human interleukin 2 receptor, only replacement of M4 had no effect on channel function, suggesting that M1 and M3 are closest to M2 (Tobimatsu et al., 1987). Shortening of M4 by two to four amino acids did not affect AChR function, al-

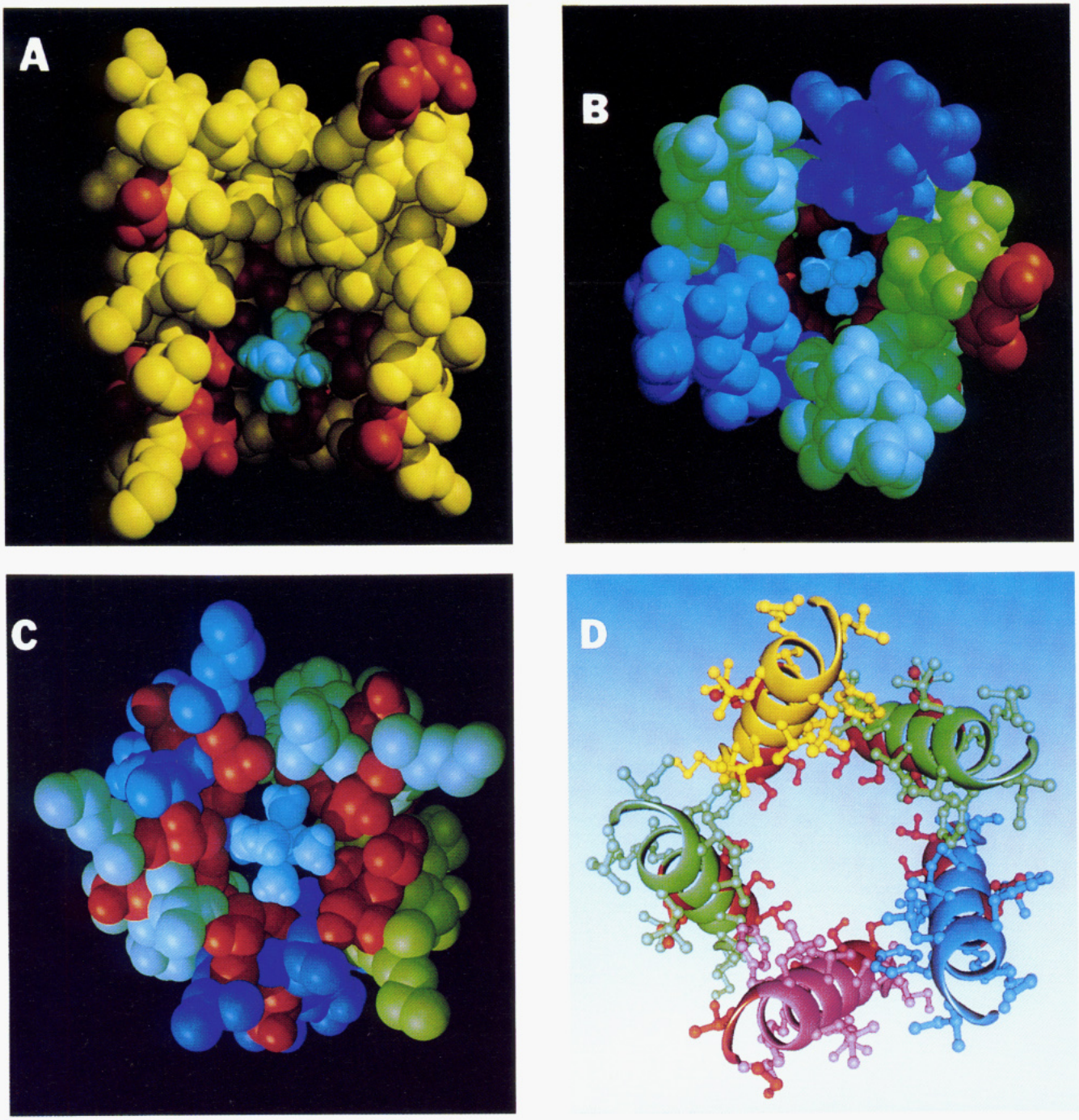

FIGURE 6: Space-filling representations of M2 sequences packed together as they may be in the central region, each rotated by 72° about the vertical channel axis and each at the same height, which have quasi 5-fold symmetry. Packing considerations with these constraints seems to favor a left-handed twist to the bundle and display a characteristic "knobs into holes" packing as optimal (Stroud & Finer-Moore 1985). This is a proposed model which summarizes the current consensus information. (A) Cross section through the channel showing serine and other polar residues in red and a hydrated sodium ion in blue. (B) Entry well at the synaptic side of the M2 bundle with a hydrated sodium ion. (C) Bundle of M2 helices seen from the cytoplasmic side. A narrower pore on this side is suggested by effects of mutagenesis on conductance. (D) Model of the M2 bundle as seen from the synaptic side showing all side chains. Polar side chains within the channel are shown in red.

though shortening by eight residues did completely eliminate agonist-induced ion flux, indicating a minimal required length of this segment of about 14 amino acids. Replacement of two cysteine residues found on MA of the *T. californica* γ subunit by serine or phenylalanine had no effect on agonist-induced ion flux, arguing against a sterically constrained location such as tightly packed membrane-spanning α helices (Pradier et al., 1989).

Peptidic Channels

Peptides provide another powerful means for testing the effects of alterations in chemical sequence and structure on conductance. With the finding that serine-containing M2 regions are implicated in the formation of the ion channel, peptides that match the sequence of M2 were synthesized and presented to membrane bilayers. Such peptides generate ion-conducting channels (Oiki et al., 1988). However, they lead to a continuum of conductances from 0 to 50 pS. Only a small fraction of openings show a conductance close to that of the AChR (40 pS) in the absence of Ca^{2+}; thus, the resultant peptide channels bear little resemblance to the physiologic channels of the parent AChR molecule. Sequences

similar in length to M2 but containing only leucines interspersed with serine do form discrete cation-conducting channels when there are alternately two and one serine per turn but form proton channels when there is only one serine per turn of helix, probably due to the smaller number of bundled helices per channel in the less amphipathic sequence (Lear et al., 1988). At least one, and probably many, strongly amphipathic sequence (including one that matches MA) with lysine and carboxylic acids of α-helical period and membrane-spanning length ($\sim$20 amino acids) forms very stable, long lived channels of discrete conductances (Ghosh & Stroud, 1990). Thus, charged residues do not impede ion translocation by binding ions tightly within the pore as might be expected. Peptidic channels are especially useful as a model for mutational effects on conductance, but peptidic models clearly do not resemble the physiologic parent channel. However, channel formation seems to be relatively easy to accomplish; gating of the channel is at the heart of the function.

Gating of the Channel

Snake neurotoxins that compete with acetylcholine bind most intimately to the α chains, at the top crest of AChR (Fairclough et al., 1983), using a large part of their 20 × 30 Å surface which overlaps the ACh site (Love & Stroud, 1986). And distances mapped by fluorescence energy transfer between ligands and a noncompetitive channel blocker suggest that the ACh binding sites lie within the vestibule, consistent with a location that is about midway from the crest to the bilayer surface (Herz et al., 1989).

How then does ligand binding, relayed $\sim$40 Å to the narrow region of the pore within the bilayer, lead to opening? At least two general ideas are plausible. In one, the subunits which surround the channel may undergo some global conformational twist that opens the structure of the helix bundle around the channel (Kistler et al., 1982; Unwin et al., 1988). Binding of ACh at interfaces between subunits (Blount & Merlie, 1989; Pedersen & Cohen, 1990) would nicely fit in with this idea. The affinity for ACh increases greatly upon the continued presence of agonist, suggesting that there is a change in structure at these sites and binding could be the trigger for the interfaces to slip vertically against one another, resulting in subunit twisting. But helix twisting in the channel does not by itself regulate the physical diameter of the narrowest region, which, assuming 11 Å between helices, would change from an inner diameter of 7.7 Å for 0° twist to 8.0 Å for a 20° twist. Thus, some asymmetry in the closed state in which one subunit serves to block the channel would be required. However, the length of the narrow portion of the channel in the closed state would be longer if the helices were parallel, and so could account for the lack of conductance. Evidence from tritium exchange argues against any significant change in secondary structure upon agonist-induced opening (McCarthy & Stroud, 1989a), while electron microscopy suggests that a small degree of twisting may accompany desensitization (Unwin et al., 1988). However, upon channel opening there is a more significant change in the transmembrane region, seen by the dramatic increase in accessibility of all subunits in their transbilayer region to a photoactivable (iodophenyl)diazirine (TID) (White & Cohen, 1988; McCarthy & Stroud, 1989b). Thus, whatever the mechanism by which the signal reaches the channel, a large alteration is seen in the packing of the transbilayer helix bundle.

A second, nonexclusive possibility is that gating is electrostatic, involving regulation of tight ion binding sites within the pore that are a part of the gate. In agreement with this idea is the finding that there are two main sites of tight ion binding four turns of α helix apart in the transbilayer region (Fairclough et al., 1986, 1988). Calcium ion binding, which competes with these ions, is markedly reduced by agonist binding (Eldefrawi et al., 1975; Chang & Neumann, 1976), and the presence of calcium reduces conductance of AChR. Subunit twisting and allosteric control over structural or electrostatic gating mechanisms within the narrow transbilayer region remain viable possibilities.

Characteristics of the AChR Family

All neuronal and neuromuscular junctional AChR subunits have several common features. Most striking are the four putative membrane-spanning stretches of hydrophobic amino acids, M1, M2, M3, and M4 of 27, 20, 20, and 19 amino acids in length, respectively (Claudio et al., 1983; Noda et al., 1983b; Devillers-Thiery et al., 1983). As presumed α helices they would be 40.5–29.5 Å in length, long enough to span the $\sim$32 Å thick hydrophobic portion of the bilayer, which is 41 Å between head groups (Ross et al., 1977; Stroud & Agard, 1979). Of all the sequence domains of the AChR subunits, these hydrophobic stretches show the highest sequence homology both between subunits and between species.

The amino terminus is extracellular, and signal sequences are cleaved from all nascent subunits. There is a highly conserved disulfide-bonded loop between Cys130 and Cys144 (Kellaris & Ware, 1989) with sequence

W-P--I-C---V--FPFD-QNCT/S

that contains the only observed site of N-glycosylation in all species of neuromuscular (but not neuronal) subunits, at N143 (Nomoto et al., 1986; Poulter et al., 1989). A conserved proline–cysteine sequence is found in M1, and all of the AChR α subunits sequenced thus far have two adjacent cysteines, C208 and C209, at or near the agonist binding site that occurs on α chain alone (Figure 3).

M1–M3, closely spaced in the sequence, are followed by a long cytoplasmic domain which contains characteristic serine and tyrosine residues that are known sites of phosphorylation (Huganir, 1987) and M4 at the carboxy terminus. Common to all sequences of AChR subunits, but not found in GABA or glycine-activated channels that conduct anions, is a long sequence of $\sim$40 residues, MA, that displays a strong periodic alternation of polar and nonpolar residues, with the periodicity of α helix (Stroud, 1983; Finer-Moore & Stroud, 1984; Guy, 1984). This strongly suggests that MA lies as an α helix in an interface between polar and nonpolar environs, such as the membrane or protein surface (Finer-Moore et al., 1989) (Figure 3); mutagenesis suggests that this sequence may be important at least for correct expression of AChR (Imoto et al., 1988).

Permutations of Subunits Tune Receptors to Different Conductances, Properties, and Functions

Conservation between species identifies several fingerprints characteristic of each subunit type and first revealed how the nervous system may alter its AChRs during development. Coexpression of genes from different species (Yoshii et al., 1987; Deneris et al., 1988) or different genes within one species of muscle and/or neuronal AChR shows that subunits can generally be substituted by subunits of the same type to produce fully functional AChR. Hybrids generated from every possible homologous substitution of *Torpedo* and mouse muscle AChR subunits all form fully functional AChR (Yoshii et al., 1987).

Properties of AChR differ in ways that are probably tuned to physiological function. In calf muscle AChR a subunit

called ϵ, most homologous to the γ subunit (Takai et al., 1985), replaces the γ subunit during calf muscle development and is most likely to be the adult form of the γ chain (Mishina et al., 1986; Gu & Hall., 1988). The adult form has higher conductance, indicating how AChRs are altered during development by changes in subunit makeup. Expressed in *Xenopus* oocytes, calf muscle AChR generated much larger whole cell currents than *T. californica* AChR (Sakmann et al., 1985). While the single channel conductances are the same, the calf AChR has longer, voltage-sensitive mean open times, associated most with the δ subunit. In *Xenopus* myotomal muscle there are at least three AChR channel types of different conductances; at least two different *Xenopus* α chains are coexpressed throughout muscle development (Hartman & Claudio, 1990).

Neuronal nicotinic AChR also shows diversity through differential expression and subunit permutation (Morris et al., 1990). Neuronal α-3 from rat PC12 cells (Boulter et al., 1986), α-2 (Wada et al., 1988), α-4, and a non-α subunit called β-2 were sequenced from rat (Goldman et al., 1987; Deneris et al., 1988) and have different distributions of mRNA shown by in situ hybridization in brain sections. All neuronal subunits sequenced thus far have the characteristic M1–M4, the conserved Cys–Cys loop, and conserved proline–cysteine in M1 (Nef et al., 1988; Schoepfer et al., 1988). In contrast to neuromuscular AChR, agonist-induced flux responses are observed in *Xenopus* oocytes injected with mRNA for single neuronal subunit types, α-4 (Boulter et al., 1987). This suggests that there are a diverse array of neuronal AChRs which differ in subunit composition, distribution, and function (Deneris et al., 1988; Wada et al., 1988).

Diversity in GABA and Glycine Receptors—A More Polar, M2-Derived Pore Conducts Chloride Ions

γ-Aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the brain. Receptors activated by glycine or by GABA conduct chloride ions. The quaternary structure of the glycine receptor is also a pentamer of α (48 kDa) and β (58 kDa) subunits (Langosch et al., 1988), though expressed in *Xenopus* oocytes (Schmieden et al., 1989) or human embryonic kidney cells (Sontheimer et al., 1989) the α subunits alone form functional oligomeric receptors. The strychnine-binding α subunit of the glycine receptor (Grenningloh et al., 1987) and the α and β subunits of the GABA receptor (Schofield et al., 1987) are homologous to AChR subunits throughout their length. To date, six α, three β, two γ, and a δ chain have been sequenced. Each of the GABA receptor subunits forms functional receptors when expressed in human embryonic kidney cells (Pritchett et al., 1988; Shivers et al., 1989). The functional properties of the GABA receptor depend on subunit composition (Verdoorn et al., 1990), and the subunits of the GABA receptor form different combinations which are expressed in specific areas of the central nervous system (Wisden et al., 1988). Very exciting is the finding that many of the positively acting benzodiazepine, barbiturate, and neurosteroid drugs and the negative regulatory β-carbolines act on different permutations of chains, giving promise for selective influence over specific subgroups of the GABA receptor family, so identifying their function in the brain.

The common-denominator model of an AChR subunit, shown in Figure 3, applies to the subunits of the GABA and glycine receptors, except for the consensus glycosylation site, which is shared only by the GABA receptor β subunits, and the adjacent, disulfide-bonded cysteines near the amino-terminal end of M1, which are only found in AChR α subunits. Serines and threonines found in the M2 region of the anion-conducting GABA and glycine receptors of AChR are found in analogous positions of the M2 sequence (Figure 5). Interestingly, the number of serine, threonine, or polar residues, about two per turn, is higher than in cation-conducting M2s where it is about 1.5 per turn. They are also richer in threonine rather than in serine. Serine and threonine side chains frequently back hydrogen bond to the carbonyls four residues behind in the helix, presenting the $\delta-$ charge on the γ-oxygen, in toward the channel. This provides advantageous charge interaction in the cation channels. However, similarly oriented threonine side chains would present the γ-CH_3 in toward the channel axis, seeming to reduce its physical diameter. The larger number of polar oxygens in the more restricted channel may serve to replace the larger number of water molecules during passage of chloride, with its larger ionic radius of 1.81 Å and higher hydration versus 0.95 Å for the sodium ion. The energies of hydration for sodium are about twice as high as for chloride ion, suggesting that the chloride channel may not favor passage of sodium where the cost of replacement by hydroxyl groups is higher.

Glutamate Receptors in the Brain Are More Distant Cousins

The main excitatory neurotransmitter in the brain is glutamate. At least three different receptor types of glutamate-activated receptors are recognized; these are the *N*-methyl-D-aspartate (NMDA), kainate, and quisqualate subtypes. Like nicotinic AChR, they conduct positively charged ions. At least three cDNAs that are 69–74% identical have been sequenced that, as homooligomers or as heterooligomers, evoke a kainate-type glutamate-activated channel when expressed in oocytes (Hollman et al., 1989; Boulter et al., 1990). Though much larger than AChR subunits at 100 kDa, with twice the mass of extracellular protein as the AChR per subunit, they are followed by three hydrophobic stretches, M1, M2, and M3, a ~98% conserved cytoplasmic domain, and M4. There is strong conservation in the Cys130–Cys143 loop (Figure 5) [see Figure 5 of Hollman et al. (1989)]. Thus the kainate and nicotinic receptors are distantly related members of the superfamily. Like the GABA receptors, these subunits are differentially expressed in different regions of the brain (Boulter et al., 1990).

Family Resemblances in a Ca^{2+} Channel

The giant-sized 565-kDa subunit of the tetrameric ryanodine receptor channel that is responsible for the release of Ca^{2+} from the sarcoplasmic reticulum also contains four hydrophobic, putative membrane-spanning regions near the C-terminus, two of which show limited sequence homology with M2 and M3 of AChR, although their polarity relative to their threading through the bilayer may be reversed (Takeshima et al., 1989). Even this small degree of homology is tremendously significant as it suggests a common function.

Topography of Binding Sites and Functional Regions

Functional sites of the AChR have been associated with specific regions of the primary sequence (Figure 3). The extracellular portions of the two α subunits provide key parts of the two high-affinity agonist binding sites, as shown by covalent labeling with agonist analogues (Damle et al., 1978; Weiland et al., 1979; Wolosin et al., 1980) near adjacent cysteines 208 and 209 (Kao et al., 1984) which are disulfide bonded to each other (Kao & Karlin, 1986; Mosckovitz &

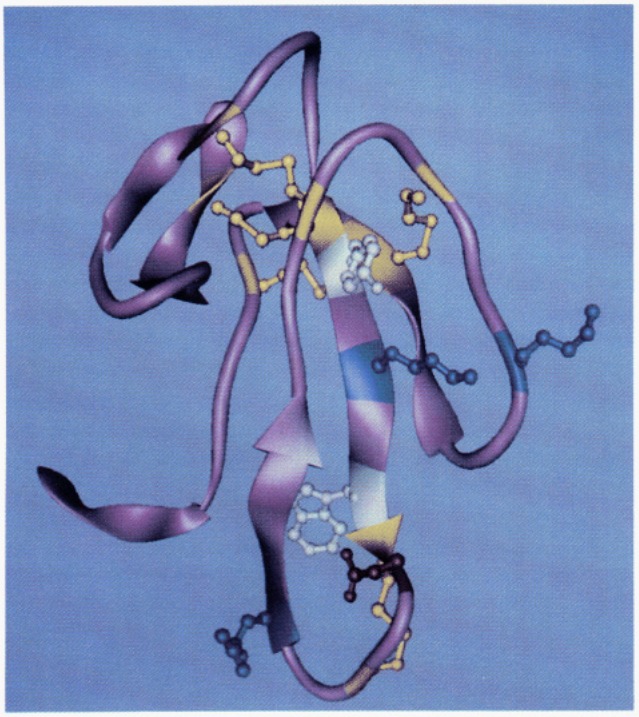

FIGURE 7: Ribbon drawing of α-bungarotoxin structure determined by Love and Stroud (1987) shows it to be an open-hand-shaped molecule. On the basis of chemical labeling and the many related sequences of toxins, a large part of the face shown is involved in the interface with AChR, and the large conformational change in AChR seen by tritium–hydrogen exchange may be due to a large refolding which occurs upon toxin binding to AChR, possibly involving reordering of the hydrogen bonding of the putative β-sheet regions that correspond to the binding sequence in AChR. α-Bungarotoxin may work by interference in the secondary structure of AChR.

Gershoni, 1988; Kellaris & Ware, 1989).

The sites of binding for antagonists presumably overlap the agonist binding site, both on the basis of their mode of inhibition and from studies of the site of covalent attachment of [^{3}H]-*p*-(dimethylamino)benzenediazonium fluoroborate, a probe that acts as both a competitive and noncompetitive inhibitor (Dennis et al., 1988). The binding of agonist is blocked competitively by antagonists such as the snake venom α-toxins, of which α-bungarotoxin is one with tightest binding ($K_d \sim 10^{-11}$ M), or *d*-tubocurarine, the active component of curare. The neurotoxins have little secondary structure and are very flexible (Love & Stroud, 1986; Basus et al., 1988). A large part of one side of the "hand-shaped" molecule is involved in binding AChR (Love & Stroud, 1986), probably stretching down into the vestibule from the top crest (Figure 7).

Regions of the antagonist binding site have been identified by binding of α-bungarotoxin to small peptides corresponding to regions of the α subunit [cf. Neumann et al. (1986), Gotti et al., (1987), Ralston et al. (1987), and Hawrot et al. (1990)]. Binding peptides span residues 189–220 and include Cys208 and Cys209 (Figure 3). But the binding surface of α-bungarotoxin extends over a 20 × 30 Å^2 area (Love & Stroud, 1986), and on the basis of cross-linking of toxin to AChR, the toxin interacts with multiple subunits (Witzemann et al., 1979; Nathanson & Hall, 1980). Replacement by serine of conserved cysteines 130 and 144 (Figure 3) caused complete loss of both α-bungarotoxin binding and agonist-induced ion flux, suggesting that this structural element is critical for the formation of a correctly folded binding site (Mishina et al., 1985). Replacing the α subunit specific C208/C209 by serine, while totally eliminating agonist-induced ion flux, only diminished α-bungarotoxin binding by 60–70%. Thus while involved in forming the agonist binding site, as indicated by agonist labeling experiments (Kao et al., 1984), C208/C209 are not crucial for the maintenance of the larger antagonist binding site.

Toxin binding to AChR evokes a large change in secondary structure of 300 amide N–H groups of the AChR, compared to very small differences between resting and desensitized states, as assayed by tritium–hydrogen exchange (McCarthy & Stroud, 1989a). This suggests a mechanism for toxin blockade in which the AChR chains are literally undone and re-form a new β-sheet pattern with the toxin chains. Smaller changes in tritium–hydrogen exchange rates are seen with nondesensitizing, noncompetitive local anesthetics (open channel blockers), suggesting that they do not perturb the overall folding of the AChR as they bind within the open channel.

AChR from snakes that carry antireceptor toxins are resistant to snake-derived neurotoxins; thus, their sequences help to identify regions that may be involved in the interaction with neurotoxins. Amino acid sequences for residues 119–222 of the α subunits of cobra (*Naja naja atra*) and water snake (*Natrix tessellata*) have been determined (Neumann et al., 1989), demonstrating 78% homology with other α subunits. A cluster of amino acids around the likely agonist and α-bungarotoxin binding site which is identical in *Torpedo*, human, chick, and mouse α chains is altered in both cobra and *Natrix*, further supporting the notion that these amino acids may interact with α-bungarotoxin.

Another important domain on the α subunit is the main immunogenic region (MIR), the epitope against which the majority of antibodies are directed in the autoimmune disease myasthenia gravis (Tzartos et al., 1982). By screening anti-MIR antibodies with peptides corresponding to different portions of the α subunit sequence, the MIR has been localized to residues 6–85 of mouse α subunit (Ratnam et al., 1986) and residues 46–127 of *T. californica* α subunit (Barkas et al., 1987), and a major component of the antigenic site has been identified as involving residues 67–76 of human α subunit (Tzartos et al., 1988). These segments, which are among the most highly charged AChR residues, must be external (Figure 3).

Polysaccharide Attachments to α Subunits Are Chemically Similar, Suggesting That Ligand Binding May Be at Intersubunit Interfaces

In AChR each subunit is glycosylated, with multiple glycosylation sites found on the γ (two to four) and δ (three) subunits (Anderson & Blobel, 1981; Nomoto et al., 1986; Claudio et al., 1989). All oligosaccharides have now been sequenced by mass spectrometry, and N143, the conserved N-glycosylation site on the α subunit, is glycosylated in all α chains, making it unlikely that differences here could explain different ligand affinities of the α chains. N453 on the γ chain between MA and M4, a consensus glycosylation sequence of some topological interest, does not carry oligosaccharide, consistent with (though not proving) a cytoplasmic location (Poulter et al., 1989).

Linkage to the Cytoskeleton

At the cellular level, AChR interacts with other components of the postjunctional membrane, including a 43-kDa protein that has actin binding capacity and several other minor components, including 300-kDa dystrophin, associated with formation and stabilization of the junctional specialization. The

43-kDa protein is cytoplasmic, colocalized with the AChR with an approximate 1:1 stoichiometry (Sealock et al., 1984; LaRochelle & Froehner, 1986). It is thought to cross-link the AChR to the cytoskeletal actin network, interacting through the AChR β subunit (Burden et al., 1983), immobilizing it to the subsynaptic region of the muscle (Sealock, 1980, 1982). The 43-kDa protein and other peripheral proteins can be stripped from postsynaptic membrane vesicle preparations by incubation at pH 11 (Neubig et al., 1979; Moore et al., 1979).

Recently, two groups applied three-dimensional image reconstruction techniques to examine the interaction of the AChR with the 43-kDa protein. Toyoshima and Unwin (1988) compare the three-dimensional structure of AChR in ordered, helical arrays both at neutral pH and at pH 11, which can loosen the attachment of the 43-kDa protein. However, the 43-kDa protein was never removed and remains associated with the AChR-containing vesicles as a result of prior treatment with *N*-ethylmaleimide (Barrantes, 1982). A region of density directly beneath the cytoplasmic extension of the AChR was observed in native preparations but was absent at pH 11. Assuming that the attached 43-kDa protein but not the cytoplasmic portion of the AChR became disordered upon treatment at pH 11, Toyoshima and Unwin (1988) interpreted this density as representing the position of the 43-kDa protein as it interacts with the AChR.

Conversely, ordered tubular vesicles of *T. californica* AChR grown both from native vesicles containing the 43-kDa protein, and grown after its complete removal by pH 11 treatment, separation of the solubilized 43-kDa protein and the AChR-containing vesicles by centrifugation, and return to neutral pH, show that the 43-kDa protein lies under the membrane and between receptors (Mitra et al., 1989). Three-dimensional reconstructions at 22-Å resolution were generated from tilted images of uranylacetate-stained AChR, and images were corrected for local lattice disorder. The information normally lost due to the limited tilt angle of grids was accounted for in part by the addition of the appropriate (001) reflections recorded by X-ray diffraction from oriented AChR membranes at a resolution of 12.5 Å. The first contour in the difference maps is 1.2 times the standard deviation of the noise, calculated as described, so that the difference density in these maps is quite clear and at least four times this noise level [see Figure 13c of Mitra et al. (1989)]. Comparison of three-dimensional reconstructions of AChR in native 43-kDa protein containing vesicles with AChR in stripped vesicles where the 43-kDa protein was removed prior to crystal growth shows that the 43-kDa protein binds to the AChR along the cytoplasmic surface of the postsynaptic membrane. This mode of attachment is consistent with the observation that the 43-kDa protein associates with lipid bilayers directly (Barrantes 1982; Porter & Froehner, 1985), presumably through its N-terminal myristoyl group (Musil et al., 1988).

Phosphorylation—A Role for Desensitization?

The physiological role of agonist-induced desensitization of the AChR has always been unclear, since at the synapse acetylcholinesterase will typically remove acetylcholine at a rate such that the AChR will not be exposed to a high enough concentration of agonist for a sufficient duration to induce even the rapid phase of desensitization. However, the phenomenon of desensitization is common to all of the neurotransmitter-gated ion channels (Katz & Thesleff, 1957; Bormann et al., 1987; Trussel & Fischbach, 1989; Tang et al., 1989) except perhaps the kainate receptor (Kiskin et al., 1986). This suggests that it might either be physiologically important or be a consequence of a mechanism in which the AChR structure relaxes as the initial low-affinity binding of ACh, whose excess binding energy can be relayed to channel opening, becomes high affinity as the binding site slowly refolds to accommodate the bound ligand. This model is analogous to action of a two-state seesaw in which the seesaw eventually bends; it would be a thermodynamic consequence of the most efficient use of binding energy in generating an unstable (conducting) state.

Phosphorylation of AChR may regulate its functional state. cAMP-dependent protein kinase from *T. californica* electric organ phosphorylates both the γ and δ subunits of the AChR (Huganir & Greengard, 1983) at single, consensus cAMP-dependent protein kinase sites (Yee & Huganir, 1987). Similarly, the β, γ, and δ subunits of *T. californica* AChR are phosphorylated by an endogenous tyrosine-specific protein kinase, also at single sites (Huganir et al., 1984). Phosphorylation in vivo was demonstrated by showing [^{32}P]ATP labeling of the AChR in rat myotubes (Miles et al., 1987) and mouse BC_3H_1 myocytes (Smith et al., 1987) at low levels in the absence of forskolin and other kinase activators and at higher levels in their presence. The first indication that phosphorylation might influence the extent of desensitization of the AChR was that the addition of a phorbol ester, which stimulates protein kinase C, to mouse or chick embryonic muscle cells increased the rate of agonist-induced desensitization (Eusebi et al., 1985). Also, in patch-clamp studies, the application of forskolin, which stimulates cAMP-dependent protein kinase, desensitized rat soleus muscle AChR without affecting channel conductance (Albuquerque et al., 1986; Middleton et al., 1986). In studies of chick embryonic muscle cells, addition of phorbol esters led to changes in conductance and average open times in addition to accelerating desensitization (Eusebi et al., 1987). This suggests that phosphorylation by cAMP-dependent protein kinase and by protein kinase C of independent sites on the AChR leads to different effects on channel function. Phosphorylation of pure, reconstituted *T. californica* AChR by cAMP-dependent protein kinase was also correlated with acceleration of the rapid phase of agonist-induced desensitization by an in vitro flux assay (Huganir et al., 1986), demonstrating that the effects of phosphorylation were maintained in the absence of other cellular elements. Coexpression of specific *Torpedo* AChR subunit mRNAs with total cat muscle poly(A)+ mRNA generated hybrid AChR which desensitized at the more rapid *Torpedo* rate (Sumikawa & Miledi, 1989). This effect could largely be correlated with the *Torpedo* γ subunit, perhaps due to the existence of a cAMP-dependent phosphorylation site on the *Torpedo* γ subunit which allowed the acceleration of desensitization by phosphorylation. Finally, in patch-clamp studies of pure AChR reconstituted into defined lipids, accelerated of desensitization by phosphorylation of tyrosine residues with protein tyrosine kinase was additive, increasing with the number of tyrosines modified per AChR (Hopfield et al., 1988). Thus, the cell can regulate the responsive state of its AChR by controlling the extent and type of phosphorylated residues. A possible first messenger which may help control this process is calcitonin gene-related peptide, a neuropeptide which has recently been shown to enhance the rapid phase of AChR desensitization reversibly in mouse soleus muscle (Mulle et al., 1988).

Amino acid analysis indicated seven phosphoserines/AChR (Vandlen et al., 1979) while an in vitro cAMP-dependent protein kinase phosphorylates single serine residues on γ and δ (Yee & Huganir, 1987). However, all of the consensus phosphorylation sites of the AChR were examined by mass spectrometry, and no phosphorylated amino acids were de-

tected even in the continued presence of sodium fluoride as a phosphatase inhibitor (Poulter et al., 1989), suggesting that phosphorylation, which occurs in vivo on the α, β, and δ subunits in mammalian cell cultures (Miles et al., 1987; Smith et al., 1987), may be labile. If the seesaw model of desensitization is correct, this apparent control over the rate of (nonphysiological) desensitization may actually reflect a functional alteration of the amount of binding energy that can be usefully transferred to channel opening, and so to mean open time and conductance.

Conformational Change upon Agonist Binding: Desensitization

The structure of ice-embedded *T. marmorata* AChR was redetermined at ~20-Å resolution in both the presence and absence of the agonist carbamylcholine by Unwin et al. (1988). Regions of density extending downward from the top of the AChR, blurring into the less well resolved cytoplasmic domain of the AChR subunits, were slightly altered in position in the presence of high (>100 mM) carbamylcholine, possibly indicating a change in the quaternary structure of the AChR. This difference was ascribed to the δ subunit tilting outward from the center, toward the neighboring γ subunit.

The conformation of AChR, as analyzed by tritium–hydrogen exchange, shows no differences between resting and desensitized AChR (McCarthy & Stroud, 1989a), indicating that the two states differ little in their overall hydrogen-bonded secondary structure and solvent accessibility. A major change in the transmembrane region of all four subunits upon desensitization is seen by agonist-induced alteration in the incorporation of the hydrophobic, photoactivated probe [^{125}I]TID (White & Cohen, 1988; McCarthy & Stroud, 1989b). The largest effect of desensitization upon [^{125}I]TID labeling is on the γ subunit, whose extracellular domain has also been shown to be perturbed upon desensitization solely on structural grounds by Unwin et al. (1988). But most importantly, the TID data suggest that ligation of the AChR causes a major change in the configuration of all subunits in their contacts with the lipid bilayer.

Perspective

Overall, the ACh receptor superfamily provides a focus for the study of many current problems in cell and membrane biology, including regulation of transcription during development, protein sorting, assembly, localization, and control over conductance and gating at the molecular level. As the best studied member of a superfamily of ligand-gated ion channels, AChR currently provides the most information about the relationship between ion channel structure and function. Molecular events between ligand binding and channel opening are still undescribed. However, an understanding of the effects of sequence on selectivity and conductivity is within reach. While a charged vestibule rationalizes gross selectivity, anions versus cations, it does not provide a way to discriminate among monovalent vs divalent ions or among similarly charged ions of different size. These finer types of selection must result from specific interactions between the permeant ions and the channel walls. Crystallization and high-resolution structure analysis by X-ray diffraction of these molecules is a key goal for furthering our understanding of ion channel function. But the understanding even now has advanced so much in the last 2 years alone that the words of C. F. Stevens "we can anticipate that the next few years will finally provide the data for a rational taxonomy of channels in the brain" (Stevens, 1987) are becoming reality.

Acknowledgments

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