Molecular biology of the acetylcholine receptor

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Abstract

The acetylcholine receptor (AChR) structure has been determined by electron microscopy and image reconstruction. The protein has also been crystallized and this 300,000 Dalton complex has been subjected to x-ray diffraction. Analysis of the sequence suggests a secondary structural pathway for the polypeptides that is common to all four gene products. Proteases have been used to cleave the protein and to identify sites of post translational modifications. Changes in conformation associated with activation and desensitization have been probed using tritium-hydrogen exchange. The large conformational changes associated with toxin binding can be understood in terms of the high resolution structure of α-bungarotoxin and the target site on the receptor.

Introduction

The acetylcholine receptor is a five subunit glycoprotein of which the structure is illustrated in Fig. 1 [1, 2]. Each subunit is transmembranous and the complex of five subunits α2βγδ encloses a central stain filled region which contains uranyl ions as seen by quantitative electron microscopy [3]. The receptor-rich membrane fractions can be annealed into tubular structures [4] and these have been used for image reconstruction [5]. All previous analysis has been of membranes which included the 43 kDa cytoskeletal proteins associated tightly with AChR (see refs. 1, 2). These proteins can be removed by treatment at pH 11 for 1 h. Such treatment reveals that little of the 43 kDa proteins remains yet the function of the receptor remains intact.
Three-dimensional image reconstructions before and after stripping of the cytoskeletal proteins reveal locations of extra density on the cytoplasmic surface of the AChR. This provides the first evidence for a well ordered association between components of the membrane and cytoskeletal components within the cell. The function of these components is not yet clear and may be connected with grouping of receptors at the densely packed synaptic region or with the binding of actin inside the cells. Protein(s) in the 43 kDa protein band has actin binding capability on Western-style blots stained with actin. Side views of the three-dimensional structure tend to be elongated perpendicular to the membrane due to an artifact present in all electron micrographic reconstructions from limited tilt angle, the problem of the missing region of data corresponding to the limitation of tilt angle of the sample produces elongation perpendicular to the membrane surface and this removes the castellated upper crest from the reconstructed image.

Subunit arrangement

The arrangement of subunits has been mapped around the receptor crest. This can be done on receptor containing tube structures using antibodies. However, the large size of the antibodies makes their sites of attachment uncertain, and often the antibody molecule will produce a large difference in comparison with the receptor

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Fig. 1. (A) Structure of the ACh receptor deduced from x-ray and electron optical studies. (b) Schematic of the helical bundles viewed from above, and suggesting the mechanism of channel formation.
structure. Consequently, isolated particles were used first to map distances between the α-chains using monoclonals directed at specific α-chain antibodies. It was proved by this means that the α-chains were 144 ± 4° apart [3]. Using the tubular structures this angle could not be measured easily because of flexibility in the antibody receptor links [6]. Using isolated molecules the centroids of the distributions associated with the positions of FAB fragments map precisely their centroid location within 3° relative to the central ion containing channel.

*Location of the gate? And possible mechanisms of gating*

Recent structural data pertain to the location of calcium ions. By replacement with terbium, calcium ion sites were localized within the central channel region of the AChR in the transmembraneous region. These bound ions may have some relevance to the understanding of gating by the AChR protein [7].

*Formation of the ion conducting channel is within helical bundles*

X-ray diffraction shows that there are α-helices oriented perpendicular to the membranes in AChR [3]. The ion channel is contained in the center of the five subunit complex. The complete sequences of all chains in several species have been determined from cDNA and genomic clones. These sequences led to a prediction of the transmembrane crossings associated with each subunit. Four very hydrophobic transmembrane crossings are found with one strongly amphipathic sequence that could lie at an interface between a polar region and a nonpolar region, perhaps at the center of the ion channel-forming structure [2, 8, 9]. To map the topology quantitatively, we made antibodies against peptide that corresponded to sequences within

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Fig. 2. Electron micrograph of the ACh receptor at the surface of a vesicle.
the mature protein [7]. Specifically, antibodies against the carboxy terminus were raised to allow determination of its location relative to the bilayer and therefore determine the number of membrane crossings in the C-terminal region. Using these antibodies, we probed the C-terminal location in three different ways, all leading to the same result. First, in sealed vesicles which are greater than 90% right side out, a radio-immunoassay shows that the available protein does not compete with radioactive peptide bound to the antibodies. Addition of saponin, however, which is seen in the electron microscope to open holes in the lipid region of the membrane not in association with protein, permit competition between the receptor and radioactive peptides. In a second approach, the antibody was coupled to protein A. This assay also indicated that the epitope corresponding to the carboxy terminus of the δ-chain became available only when sealed vesicles are treated with saponin or solubilized in the detergent sodium cholate. A third assay, immuno-electron microscopy carried out on minimally disrupted native tissue showed that the anti-carboxy terminus antibodies bind on the cytoplasmic surface of the membrane. This is the most powerful evidence that the C-termini of chains are localized on the cytoplasmic side of the membrane [8]. Nevertheless, there is some uncertainty concerning the location of the C-terminus since a disulfide bond formed between pentameric molecules via cysteine residues in the δ-chains are thought to cross-link C-

Fig. 3. Wooden model representing the subunits of ACh receptor as they surround the ion channel.
termini together. Such a cross-link is most likely to be formed on the outside of the cell and not on the cytoplasmic side. Furthermore, access to reducing agents shows more rapid reduction by a membrane impermeant reducing agent upon addition of detergent. This suggests, but does not prove, that the disulfide is external. Proteolytic evidence suggests that the peptide including this disulfide is at the C-terminal. Until these issues are resolved, therefore, there is sufficient reason to question any antibody based approach to topographic mapping of sequences.

Chemical modification sites and topography

In an approach designed to avoid the difficulties associated with topographic mapping, we sought to identify chemical labels attached to the protein from either side of the membrane. Peptides generated from the sequence were introduced into a liquid secondary ion mass spectrometer and identified according to their molecular masses [9]. This made it possible to identify sites of modification in the protein. Initially these experiments are focussed on intrinsic modifications, glycosylation and phosphorylation, which occur in the native protein. Since masses of each peptide can be determined with a precision of 1 proton up to a total mass of about 3,000 Daltons, many peptides have already been mapped and assigned to regions of the amino acid sequence. This makes it possible to identify those peptides which have been chemically modified [9].

We have used these mass spectrometric approaches to identify all of the sugars associated with AChR. Furthermore, we assigned structures to all the polysaccharides released by treatment with PNGaseF. One molecular mass peak establishes that asparagine 144, conserved in all chains, carries an N-linked polysaccharide. It is clear that all α chains carry this modification uniformly, thus any difference between the behavior of the α-chain cannot be associated with whether or not polysaccharide is attached to one or other of the chains since both carry the modifications [9]. One informative site of potential N-glycosylation lies in the region between M5 and M4 in the γ-chains of *T. californica* AChR [2, 10]. This loop may lie either on the external surface or the cytoplasmic surface of the plasma membrane according to most recent data. By identifying this peptide we established that it is not post-translationally n-glycosylated. Therefore, its location relative to the cytoplasm or outside of the membrane remains unknown. Nevertheless it is clear that this powerful technique can be used with extrinsic chemical modifications to determine topography of the chains.

From site-directed alteration and from substitutions of sequences in the gene coding for α-chains, Numa and his colleagues showed that M5 appears to be important to obtaining expression of the AChR on the cell surface; M4 could be replaced by a hydrophobic helix from other sources and yielded functional receptor. Thus at present no clearly defined role can be assigned to M5 though it still may form part of the channel, and it may lie in the surface of the membrane or the molecule, probably on the cytoplasmic side of the membrane.
At least two peptides from ACh receptor make ion conducting channels

To see if a peptide corresponding to the sequences of M5 and of M4 would themselves be membrane active or channel-forming in association with a lipid bilayer, these sequences were synthesized. The 26 amino acid sequence corresponding to each of these two regions of the β-chain (MA) or the α-chain (M4) were generated by solid phase peptide synthesis. When associated with phospholipid bilayers on the surface of a patch pipette, discrete currents were recorded from the peptide corresponding to MA after 15 minutes [11]. So far neither the stoichiometry of peptides per channel nor any details of the structure have been determined. The presumption is that a bundle of helical peptides present a polar surface to the center of a channel formed across the membrane. The hydrophobic sides presumably interface directly with the lipid regions. These channel recordings remain open for somewhat longer periods of time than in the native receptor, nevertheless ion-conducting channels are formed [11]. At the same time it was found by Montal that M2 was capable of forming ion-conducting channels with similar conductances and rather shorter lifetimes. We therefore conclude that amphipathic peptides are membrane active and capable of forming channels. However, these channels must be very different from those formed physiologically since two sequences from the same molecule seem capable of forming ionic channels. We presume that there will be many peptides from soluble proteins or amphipathic peptides that could be designed that are also equally capable of making ion-conducting channels with square wave conductances. This approach is therefore not capable of mimicking the physiological channel and is one we have elected not to pursue further. The phenomenon is undoubtedly interesting though interpretation of the results is open to much speculation and little direct proof.

Dynamics of change in structure of the receptor upon ligand binding

Tritium exchange experiments show that there are conformational changes which accompany channel opening though these appear to be small in comparison with the effects of toxin or curare binding [12]. By this technique, desensitized receptor seems to have the same exchange characteristics as does the native resting receptor. In related attempts to understand the nature of the conformational change which could produce ion gating in the AChR channel, we studied the binding of elements that replace calcium [7]. Terbium Tb³⁺ binds to the AChR competitively with calcium ions. Approximately 50% of these ions are displaced by the binding of agonists suggesting that they are in some way linked to the conformational changes that accompany opening. We were able to locate the terbium ions by anomalous x-ray dispersion, a very precise technique since the signal obtainable at a tuneable x-ray source, in this case the SSRL synchrotron in Palo Alto, provides a 20% difference in the scattered radiation. Several main loci of terbium sites were found; two
of these were within the span across the bilayer region of the x-ray diffraction profile. Thus these ion binding sites are presumably in the ion-conducting channel forming region in the center of the complex of five subunits. This suggests that an electrostrictive gating mechanism may be in effect and that components of the gate may control the binding of calcium ion within the channel-forming regions. This could explain why gating by the binding of ligand on the outside surface of the AChR can control ion binding a long distance (750 Å) away without any global change in configuration. A small change could be relayed to the electrostrictive site [8].

Neurotoxins may work by unzipping the β-sheet structures

Neurotoxins like α-bungarotoxin bind the AChR at sites that have variously been identified with the peptidic region between residues 130 and 220 of the α-subunit. We determined the three-dimensional high resolution x-ray structure of α-bungarotoxin and found it to contain extensively β-sheet regions between fully extended peptide chains [13]. This flexible, open-hand shaped molecule binds to the top crest of the AChR as determined by x-ray diffraction [3]. Since the predictions for this part of the receptor are that it contains β-sheet structure [3], the suggestion is that its mode of action as a neurotoxin is to undo the β-strands within the external surface of AChR and form new β-sheets between the toxin and receptor. This extensive change could account for the tritium exchange data and explain why the irreversible blockade is evoked [12]. The chains are simply unwound and no longer can cooperate in ligand binding or channel opening. Each proton resonance in the structure has been assigned by two-dimensional NMR [14, 15], thus permitting a better assessment of the dynamics of association of this tight complex.

While unambiguous assignment of chemical attachments is now possible in this complex molecule, and while site-directed mutants have drawn attention to certain sites as more or less important or more or less connected with the channel-forming region, the knowledge of structure requires an additional, more direct three-dimensional approach. X-ray crystallography is so far the only known technique capable of revealing the three-dimensional folded structure of a protein of this size. Electron diffraction methods which we have used to obtain 3.5 Å resolution projected structures for bacteriorhodopsin, for example, are unlikely to reveal this internal structure without several new developments [16]. Thus we have chosen to focus now on interpretation of the three-dimensional crystal structure of AChR using crystals that have now been grown [17]. Coupled to high resolution mass spectrometry as a means of determining exact chemistry of native and modified proteins, we expect that functional determinants of structure can subsequently be identified.

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