STRUCTURE AND FUNCTION OF AN ACETYLCHOLINE RECEPTOR

JOERG KISTLER, ROBERT M. STROUD, MICHAEL W. KLYMKOWSKY, ROGER A. LALANCETTE, AND ROBERT H. FAIRCLOUGH
Department of Biochemistry and Biophysics, University of California, San Francisco, California 94143 U.S.A.

ABSTRACT Structural analysis of an acetylcholine receptor from *Torpedo californica* leads to a three-dimensional model in which a "monomeric" receptor is shown to contain subunits arranged around a central ionophoretic channel, which in turn traverses the entire 110 Å length of the molecule. The receptor extends ~15 Å on the cytoplasmic side, 55 Å on the synaptic side of the membrane. The α-bungarotoxin/agonist binding site is found to be ~55 Å from the entrance to the central gated ion channel. A hypothesis for the mechanism of AcChR is presented which takes into account the structural and kinetic data, which is testable, and which serves as a focus for future studies on the agonist-induced structure change in AcChR.

INTRODUCTION

The release of neurotransmitters from nerve terminals induces a selective increase in permeability to cations and subsequent depolarization of the electric potential across the postsynaptic membrane. This excitable membrane has been extensively characterized at the electrophysiological, cellular, kinetic, biochemical, and structural levels. Most such characterization pertains to nicotinic receptors for acetylcholine (AcCh) in vertebrate neuromuscular junctions and in the electrocytes of *Torpedo* (a marine elasmobranch) or *Electrophorus* (a fresh water teleost) (reviews, 1–4).

We present here our recent results on the structural analysis of acetylcholine receptor (AcChR) in *Torpedo californica* electrocyte membranes. These and related results are discussed with reference to possible mechanistic consequences, focusing on a testable hypothesis for AcChR action.

AcChR-rich membrane fractions are routinely obtained by differential centrifugation of homogenized electric organ and subsequent separation on density gradients (5). Radioactively labeled neurotoxins such as α-bungarotoxin, which bind extremely tightly but noncovalently to AcChR with dissociation constants in the range 10^{-10}–10^{-11} M at room temperature (6, 7), serve as a marker through the isolation procedure; agonist-dependent cation flux can be measured for microsac preparations to ensure functional integrity of AcChR in vitro (8, 9). Under reducing conditions SDS polyacrylamide gel electrophoresis of such preparations shows six major bands with apparent mol wts of 40,000 (α), 43,000, 50,000 (β), 60,000 (γ), 65,000 (δ), and 90,000 (10). Exposure to pH 11 for 1 h at 20°C removes the 43,000 and 90,000 mol wt polypeptides from the AcChR-rich membranes without loss of agonist induced fluxing ability (10, 11). These chains are therefore presumed to be peripheral membrane proteins. Functionally intact AcChR vesicles which contain only α, β, γ, and δ subunit types have been reconstituted from AcChR either solubilized in octylglucoside (12) or in the presence of exogenous lipids and cholate (13, 14). The subunit composition of monomeric AcChR has been established independently in several laboratories (15, 16) and found to be $\alpha_2\beta\gamma\delta$.[1] In vivo, however, AcChR occurs as a dimer cross-linked via a disulfide between δ subunits (17, 18), though both the monomeric and dimeric forms show identical ligand binding affinity and are equally active in ^{22}Na-flux assays (19). Thus, the physiological relevance of dimerization is as yet unidentified.

Evidence in favor of discrete ionophoretic channels in the postsynaptic membrane was first obtained from the noise-analysis of agonist-induced current fluctuations measured for motor endplates under voltage-clamp conditions (20, 21). More recently, with improved techniques elementary single channel events have been directly observed as all-or-none, rectangular, pulse-like wave forms of constant amplitude (22). Agonist, temperature, and membrane potential determine the average channel lifetime to be typically 1–3 ms. At 20°C ~ 10^4 Na^+ ions flow into the cell during the opening of a single channel, induced by the binding of AcCh. The ionophoretic channel conducts ions as large as dimethyldiethanolammonium and must therefore be at least 6.5 Å Diam (23) throughout its entire length in the "open channel" state.

Please address correspondence to Dr. Stroud.

[1] First proposed by Reynolds and Karlin (47).

Which subunit contains the agonist binding site and therefore acts as the primary effector of the protein conformational change associated with the opening of the transmembrane channel? Using a variety of affinity labels and neurotoxins, the AcChR agonist (or antagonist) binding sites have been located on the two α subunits (review, 4). Following reduction with dithiothreitol, one α subunit of the *T. californica* AcChR can be affinity labeled with 4-(*N*-maleimido)-benzyltrimethyl-ammonium (MBTA) or with bromoacetylcholine (BAC), either of which react covalently with a single sulfhydryl and block 50% of the available toxin binding sites (24, 25). Delegeane and McNamee (26) claim that after one AcCh site is blocked by alkylation with MBTA ^{22}Na-flux is still induced by binding of carbamylcholine to the second site. However Lindstrom et al. (14) showed, using reconstituted AcChR vesicles, that alkylation with MBTA in fact removed all agonist dependent fluxing ability. Both results indicate that ligand binding to one particular α-subunit (and not the other) leads to channel opening. In unmodified AcChR it is possible that agonist binding to both sites is more effective than simply to the one site (27). The Hill coefficient for agonist-induced ion permeability response is 1.97 ± 0.06[2] thus two agonists are required for activation.[3,4]

AcChR IS FUNNEL-SHAPED: ALL SUBUNITS SPAN THE BILAYER

The transmembrane nature of the AcChR complex has been independently demonstrated by x-ray diffraction studies and by use of analytical probes such as proteases or anti-AcChR-specific antibodies. Analysis of continuous x-ray scattering profiles recorded from pellets of AcChR-rich membrane preparations revealed an asymmetric distribution of protein perpendicular to the lipid bilayer, and showed that the protein extends 55 Å on one side of the membrane and 15 Å on the other (28). In conjunction with the in-plane dimensions of AcChR from electron microscopy and the measured density of receptors per unit area, this analysis provided a low-resolution, cylindrically averaged structure of AcChR (29).

A sharp reflection of spacing 5.1 Å is oriented exclusively perpendicular to the membrane plane. This is the expected angle for scattering from α-helices alone, and circular dichroism suggests that 34% of AcChR is α-helical (30). The sharpness of the x-ray reflection indicates that the α-helices in AcChR are on average 80 Å long; its orientation implies that essentially all α-helices are almost perpendicular to the membrane, and this is strong evidence that some (at least two) or all of the subunits are themselves elongated perpendicular to the membrane plane.

The funnel-shaped structure of AcChR is directly visualized in side view by negative stain electron microscopy at the edge of a folded-over membrane vesicle (Fig. 1). Proof that these structures are membrane-bound AcChR oligomers was obtained by immuno-electron microscopy, since we were able to label them with anti-AcChR antibodies coupled to ~200 Å-sized colloidal gold spheres (29). Furthermore, experiments in which AcChR was treated first with α-bungarotoxin and then with anti-toxin antibodies attached to gold beads established that the protein protrusion of 55 Å was on the extracellular, i.e., synaptic, side of the membrane. The smaller protrusion on the cytoplasmic surface was more difficult to visualize by electron microscopy. However, as membranes dry down onto the carbon support film, folds are often formed. A small portion of these are sharp, and unambiguously show the extracellular 55 Å protrusion of AcChR molecules on the outer surface of the folded double membrane (Fig. 2). The minimum overall width of such folded double membranes measures ~200 Å, close to twice the 110 Å length of the AcChR oligomer determined by x-ray diffraction analysis (slight shrinkage is likely to be associated with specimen dehydration for electron microscopy). The central double bilayer thickness averages 86 Å. As this value is close to twice the 40 Å thickness of the single bilayer (28), there must be little of the protein protruding from the cytoplasmic surface. This observation is in agreement with the asymmetric protein profile computed from the x-ray diffraction data. The transmembrane nature of AcChR has further been demonstrated by immuno-electron microscopy: antibodies raised against solubilized AcChR and coupled with ferritin bind to both the synaptic and the cytoplasmic sides of open membrane vesicles (31, 32).

We now ask whether all five subunits span the bilayer or whether there are components which are entirely buried in the membrane while others are accessible from one side only. As each subunit type present in membrane-bound AcChR is accessible to degradation by exogenous proteases (33–35) they must all protrude into the aqueous phase and none is entirely buried within the lipid bilayer. This conclusion is further substantiated by the finding that all four subunit species can be iodinated in the presence of lactoperoxidase (36). The fact that all subunits naturally occur in their glycosylated form implies their exposure on the synaptic membrane side (37, 38). Photoactivable reagents that partition into the lipid phase of the

[2]Neubig R. R., and J. B. Cohen. 1980. Permeability control by cholinergic receptors in Torpedo postsynaptic membranes. Agonist dose-response relations measured at second and millisecond time scales. *Biochemistry.* 19:2770–2779.

[3]Cash D. J., and G. P. Hess. 1980. Molecular mechanisms of acetylcholine receptor-controlled ion translocation across cell membranes. *Proc. Natl. Acad. Sci. U.S.A.* 77:842–846.

[4]Sine S. M., P. Taylor. 1980. The relationship between agonist occupation and the permeability response of the cholinergic receptor revealed by bound cobra α-toxin. *J. Biol. Chem.* 255:10144–10156.

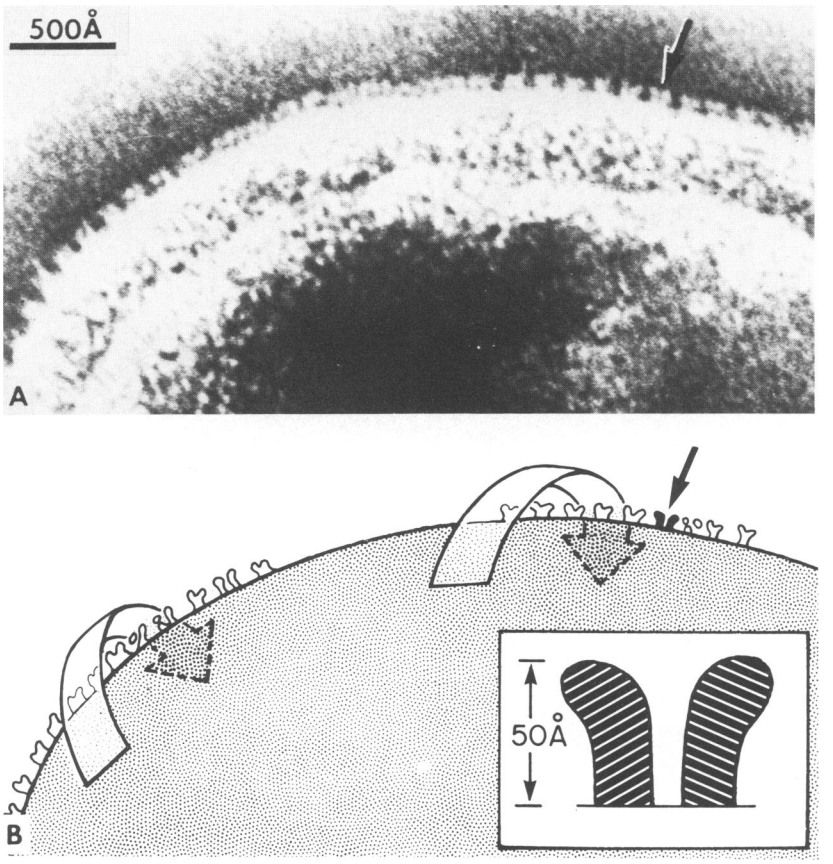

FIGURE 1 View of a uranyl acetate-stained synaptic edge of AcChR membrane vesicle (*A*) and schematic representation of the 55 Å-long funnel-shaped protrusions of the receptor molecules (*B*). The central channel is filled with uranyl stain down to at least the level of the lipid bilayer. Modified from Fig. 4 in reference 29.

membrane label at least the β, γ (39) and the α subunits (40); therefore, an exclusively peripheral location of these (and probably all) subunits is excluded. Combination labeling and proteolysis showed the transmembrane nature of the 40, 50 and 66 kilodalton chains in *T. marmarota*.[5] Recently, Strader and Raftery (35) demonstrated that all AcChR subunit types can be proteolytically cleaved from both the cytoplasmic and from the synaptic side of the membrane. Thus current evidence shows that all five subunits are elongated perpendicular to the membrane, are in contact with the lipids for part of their length, and protrude on both sides of the bilayer.

PROOF THAT EACH AcChR MONOMER IS A SINGLE INFUNDIBULIFORM STRUCTURE

Electron micrographs of negatively stained AcChR membranes (Fig. 3 *A*) reveal "rosette" structures 85 Å Diam dispersed in the plane of the membranes (29, 41–44). Each rosette is a projection perpendicular to the membrane surface of a single funnel-shaped structure. AcChR occurs in vivo as dimers of the five subunit complex; however, until recently direct evidence on whether the infundibuliform structure corresponds to a monomeric or dimeric receptor complex has been lacking.

Standard membrane preparations rich in monomeric or dimeric AcChR appear indistinguishable from each other because the molecules are so closely packed together. Membranes reconstituted from exogenous lipid and solubilized AcChR that was predominantly dimeric (79% by integration of stained bands on SDS gels) revealed rosettes spaced much further apart: 69% of the rosettes are seen to be dimerized and 31% are single rosettes (total counted, 690). In a second approach, excessively base-treated (pH 11.5 for 1 h at 20°C) and sonicated membranes from the same source (79% dimeric) also led to a portion of membranes with more dispersed AcChR mol (Fig. 3 *B*). In this case, 78% of rosettes were in pairs, 22% were single (total counted, 803). This unambiguously shows that each infundibuliform structure is one AcChR monomer. This same conclusion which we deduced in reference 28 and 29

[5]Wennogle L. P., J-P Changeux. 1980. Transmembrane ?????????? of proteins present in acetylcholine receptor-rich membranes from Torpedo marmorata studied by selective proteolysis. Eur. J. Biochem. 106:381–393.

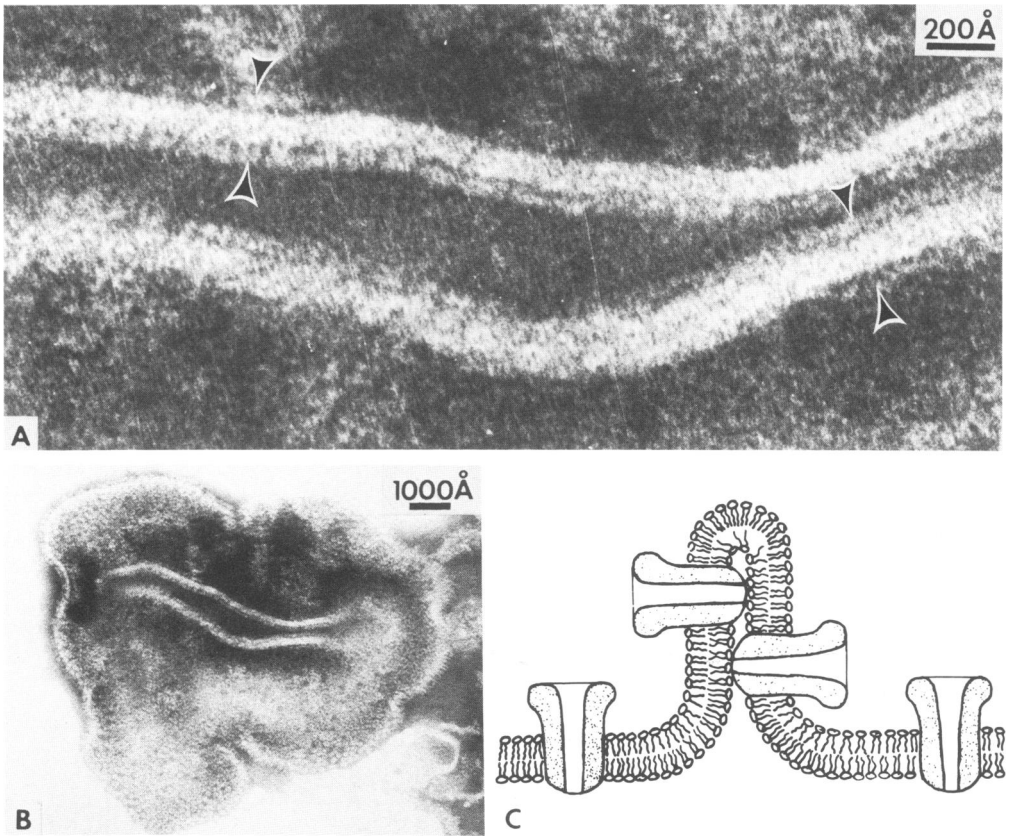

FIGURE 2 Folds of a negatively stained (uranyl acetate), synaptic side-out vesicle (*A, B*) show the two lipid bilayers in close apposition on the cytoplasmic side as depicted in cross section as if cut vertically between pairs of arrows shown (*C*). Arrows indicate regions of sharp bends of the kind where distances between protrusions and the width of the double bilayer were measured.

has been drawn from dimer-reconstituted membranes (45)[6] and for solubilised AcChR dimer (34).[7]

EACH MONOMER CONTAINS A 110-Å LONG CENTRAL TRANSMEMBRANE IONOPHORETIC CHANNEL

The centers of the rosettes generally appear more heavily stained relative to membrane regions between AcChR mol. However, quantitative analysis of the depth to which the central well is stained relative to surrounding protein-free membrane requires dispersed molecules as described in the previous section. Furthermore, since the well narrows to < 10 Å, the analysis must be carried out at high resolution, i.e., > 10 Å resolution. Image reconstructions of crystalline arrays (see below) cannot be used for such purposes because disorder in the lattice limits the resolution to about 20–30 Å. Thus, electron micrographs of membranes with widely dispersed receptor molecules, such as those shown in Fig. 3 *B*, were densitometered with a raster size equivalent to 2.4 Å. Optical densities (ODs) were averaged over 3 × 3 arrays of picture elements and overall OD distributions in stain-excluding protein regions ($\overline{OD}$ = 1.84 ± 0.01), in protein-free membrane areas ($\overline{OD}$ = 1.62 ± 0.01), and in the stain penetrated depressions ($\overline{OD}$ = 1.48 ± 0.06), are displayed in Fig. 4. OD on the film is linearly related to stain thickness in the sample. Arbitrarily setting stain thickness of the protein stain-excluding region to 0 Å, the stain thickness of the membrane bilayer region must then be 70 Å (total receptor height = 110 Å; bilayer thickness = 40 Å). Hence the stain thickness of the central depression can be determined by an extrapolation of a plot of mean regional OD vs. the known relative stain thicknesses. From this extrapolation we obtain the result that the central depression stain thickness is 114 ± 19 Å. This means that stain penetrates throughout the entire transmembrane length of the receptor at a single central ion channel under these conditions.

A THREE-DIMENSIONAL MODEL OF AcChR

We have analyzed the structure of AcChR by optical and computer filtration of two forms of crystalline membrane-

[6]Cartaud J., J-L. Popot, and J-P Changeux. 1980. Light and heavy forms of the acetylcholine receptor from *Torpedo marmorata* electric organ. *Fed. Eur. Biochem Soc.* 121:327–332.

[7]Wise, D. S., B. P. Schoenborn, and A. Karlin. 1981. Structure of acetylcholine receptor dimer determined by neutron scattering and electron microscopy. J. Biol. Chem. 256:4124–4126.

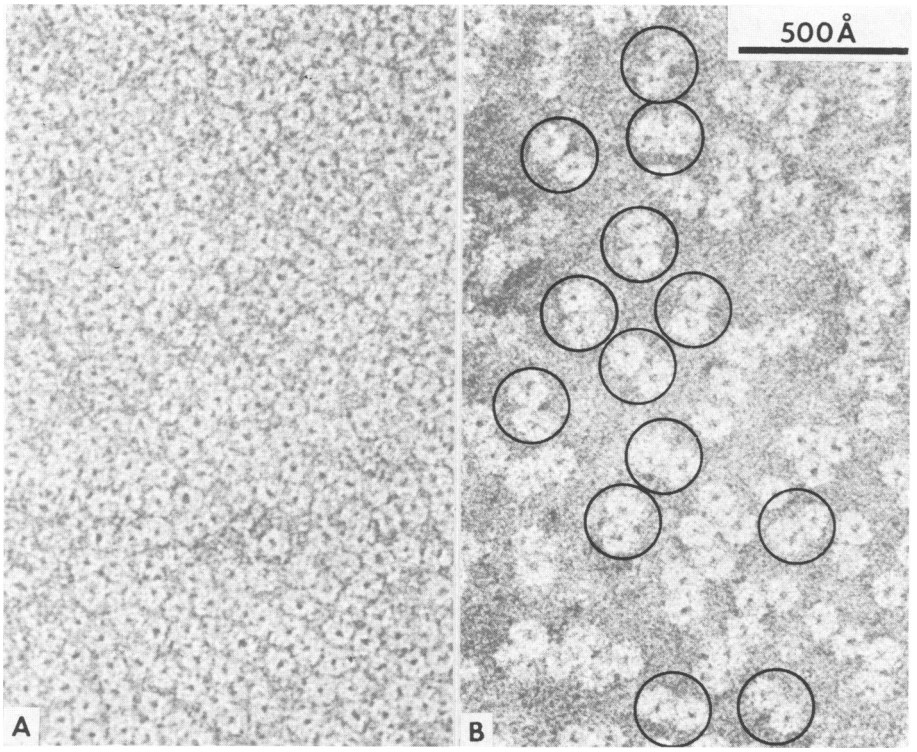

FIGURE 3 Uranyl acetate-stained AcChR membrane sheets derived from preparations rich in dimeric AcChR. In a portion of the membranes the densely packed AcChR molecules (*A*) have been dispersed by excessive base treatment and sonication (*B*). Circles outline some of the dimeric pairs of AcChR in the membrane.

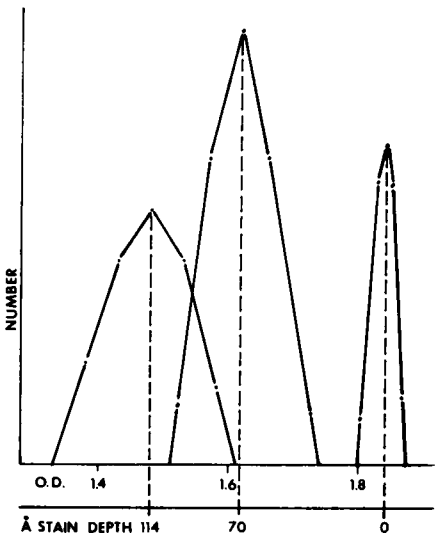

FIGURE 4 Distribution of optical densities for the stain (uranyl acetate)-filled depression of AcChR molecules (left), for protein-free membrane regions (40-Å stain excluding thickness, middle) and for the stain-excluding protein protrusion of AcChR (mean stain-excluding thickness 110-Å, right). From this graph the stain depth in the center of the infundibuliform AcChR molecule is calculated to be 114 ± 19 Å. The error is based on variance in ΔOD between individual AcChR crest and central well OD and on variance of their average values.

bound AcChR: large membrane sheets (28), and membrane tubes (45). Whereas planar crystalline arrays occur only rarely in fresh AcChR membrane preparations, ordered synaptic side out, tubular arrays are formed spontaneously at 4°C upon annealing over a period of six weeks or longer. No proteolytic degradation was observed after much longer periods under the same conditions, and the tubes were shown to contain all four subunit types of AcChR by immuno-electron microscopy (45). These structures present many differently angled views of AcChR, which resolve into the characteristic infundibuliform shape —doublets with 55 Å protrusion— at the edge (Fig. 5 *A*). Computer filtrations, viewed from the cytoplasmic surface of the membrane (Fig. 5 *B*), uniformly show a characteristic arrangement of two large peaks, one smaller peak, and a deep groove, always in the same sequence in the anticlockwise direction, and around the central ionophoretic channel. A three-dimensional model based on the scaled contour heights in filtered images and the electron density determined by x-ray analysis is shown in Fig. 6. With the partial specific volume of 0.74 $cm^3\ gm^{-1}$ calculated from the amino acid composition of AcChR (46), the total volume of the model would correspond to a total mol wt of 277,000 ± 5,000. This is somewhat larger than the measured value of Reynolds and Karlin (47) of 250,000, and the radius of gyration computed from the model is 43 Å which is slightly smaller than the value 46 ± 1 Å measured by Wise et al. (48) using neutron diffraction.

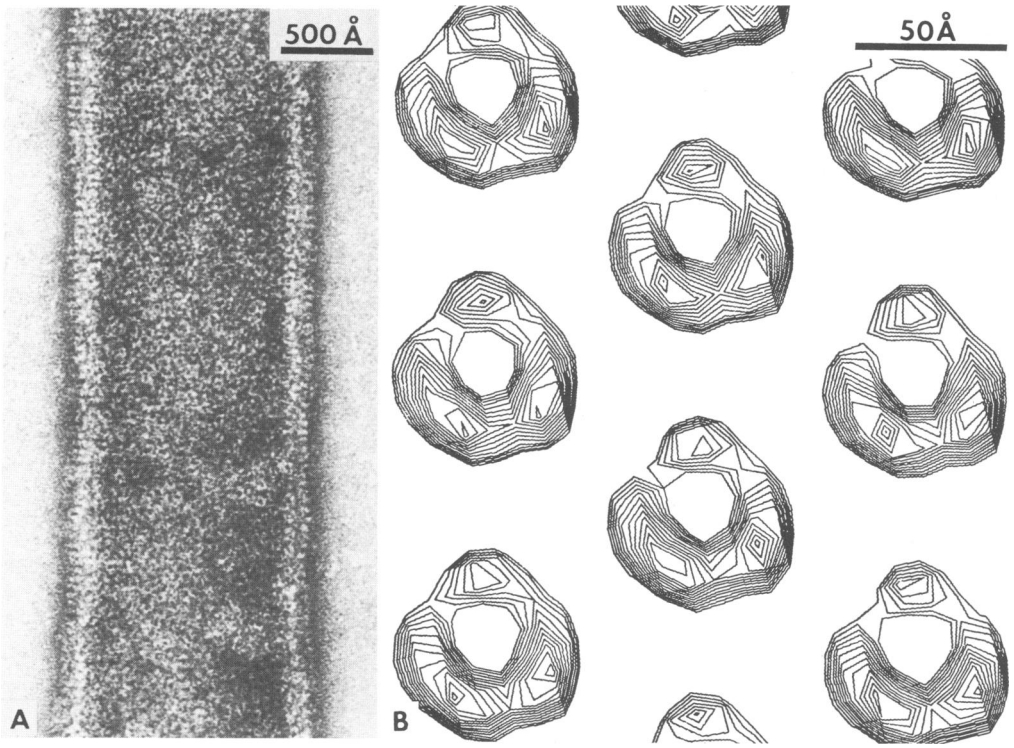

FIGURE 5 Membrane tubes with a crystalline surface lattice of AcChR oligomers spontaneously anneal from AcChR membrane vesicles. In a negatively stained specimen both sides of the flattened tube are visualized superimposed (*A*) but can be separated by computer filtering (*B*). Two major and a minor protein peak and a stain-filled groove always surround the central depression in the anticlockwise sense viewed from the cytoplasmic side (uranyl acetate stained).

In the model the individual elongated subunits must be arranged around the central channel much as five staves of a barrel. Tentative location of the subunits within the model at this stage is based on our preliminary cross-linking studies, on peak heights in the model, and on the ratios of subunit molecular weights.

NEUROTOXINS AS PROBES FOR THE AGONIST BINDING SITE

The dissociation constant for AcCh to AcChR is $\sim 10^{-7}$ M and binding is competitive with curare and curaremimetic snake neurotoxins whose dissociation constants are three to four orders of magnitude lower (49). The affinity label MBTA competes stoichiometrically with both agonists and neurotoxins (4), facts which strongly argue that toxin and agonist binding sites on AcChR overlap. Using sequence information available for α-bungarotoxin (50), we detail current knowledge of this toxin-AcChR interface, and the status of direct and indirect evidence that this regulatory site is on the synaptic crest of AcChR, far from the gated part of the channel.

The structure of α-bungarotoxin, determined by x-ray crystallography (51; see reference 68),[8] reveals that this toxin, like those of the evolutionarily-related erabutoxin *b* (52–55) and α-cobratoxin (56), is a flat "hand-shaped" molecule consisting of three disulfide cross-linked loops of polypeptide chain. Chemical modification of its various reactive amino acid side chains reduces, (but rarely abolishes) its binding affinity to AcChR (57). The reactive residues (with exception of Trp 29; see below) as well as most of the evolutionarily conserved, titratable side chains (50, 58) are dispersed on a 20 × 30 Å area on the concave surface of the molecule (see Fig. 7). Thus all current evidence points to an extended toxin binding surface on the AcChR, as Low suggests (53), rather than a single, specific binding site.

Comparison of α-bungarotoxin with erabutoxin *b* shows that the detailed secondary and tertiary structures are different. Thus either or both would have to refold in binding to AcChR to reach congruence for the positions of the interface residues. For example, there are only four inter-chain hydrogen bonds in α-bungarotoxin (Fig. 7), whereas 70% of erabutoxin *b* is β sheet. Furthermore, the side chain of conserved Trp 29 must clearly lie in the interface region. However, it is found on the opposite side of the molecule in the two toxins. Thus, the toxins (and/or the receptor) must refold upon binding as suggested by the extremely slow association rates for "long" toxins $t_{1/2}$ = 1–3 min (59) and the fact that association (but not dissociation) rates decrease rapidly below 11°C (57, 60).

[8]Agard, D. A., S. A. Spencer, and R. M. Stroud. Evolution of a molecular surface interaction: the structure of α-bungarotoxin. *Proc. Natl. Acad. Sci. U.S.A.* Submitted for publication.

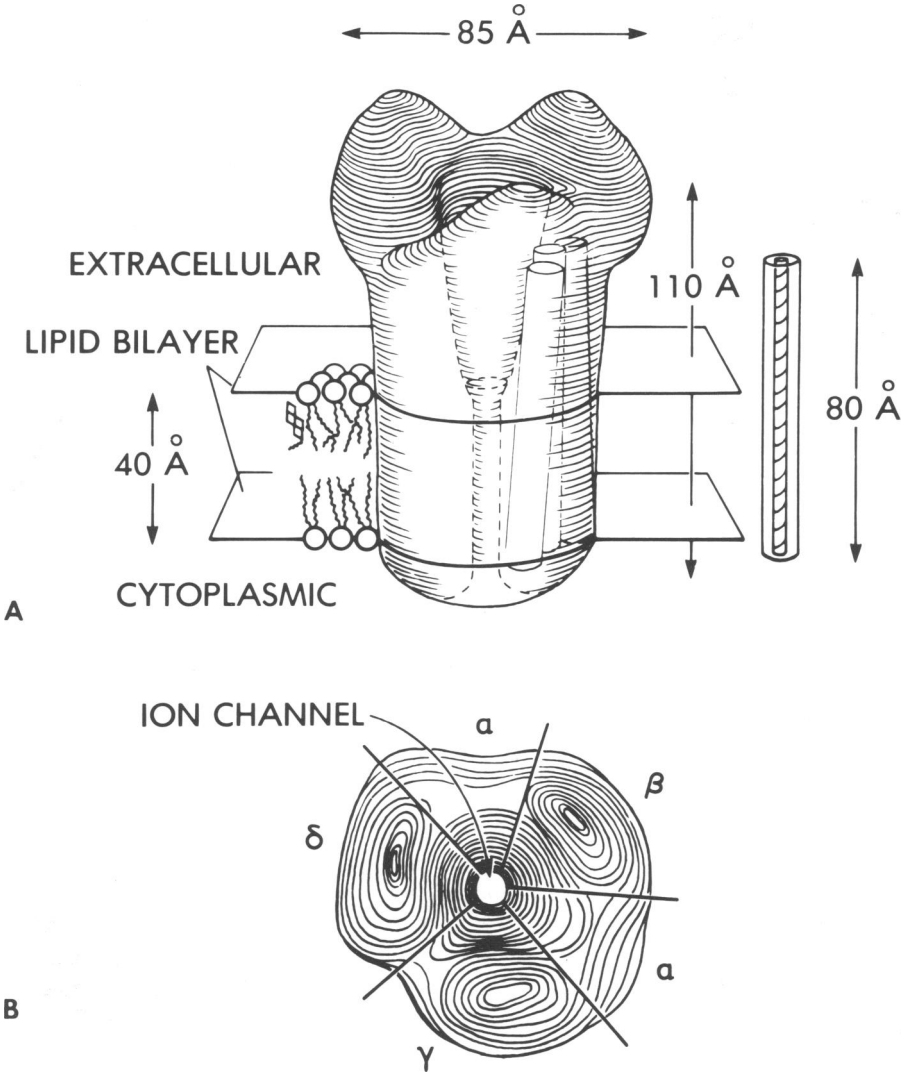

FIGURE 6 A three-dimensional model for the funnel-shaped AcChR molecule in the lipid bilayer. (*A*) On average 80 Å-long α-helices indicate an elongated shape of subunits which are arranged perpendicular to the membrane channel. The protein topography has been inferred from the densities in computer filtered images of AcChR tubular lattices, from side views, and from x-ray diffraction. (*B*) AcChR molecule viewed from the synaptic side with borders arbitrarily drawn between elongated subunits and tentative assignment of subunit types consistent with our cross-linking data which show prominent α–γ and γ–δ linkages (disuccinimidyl tartarte, 6 Å span). Karlin and colleagues (*Proc. Natl. Acad. Sci. U.S.A.;* in press) have found an average angle of 113° ± 33° between the two α subunits per monomer, in agreement with our assignment.

Based on chemical modifications (53, 57), it has been suggested that the side chains of Asp 31, Arg 37, Gly 38 probably occupy the acetylcholine binding site (53, 55). However, unlike AcCh, the toxins do not lead to channel opening. Could the extended binding interface serve to "lock" the quaternary structure of AcChR?

α-Bungarotoxin binds on the outside of the 55 Å portion of AcChR on the synaptic membrane surface. This has been directly visualized by electron microscopy of AcChR membranes treated first with toxin and subsequently with antitoxin antibodies coupled with gold beads (29). A more precise localization of the bound toxin on the AcChR molecule by electron microscopy and image processing of AcChR arrays has not yet been possible as toxin binding appears to destroy the lattices in membrane sheets or tubes. However, available evidence strongly indicates that the binding area of α-bungarotoxin is located on the top synaptic crest of the AcChR oligomer. (*a*) X-ray diffraction from oriented pellets of conventionally prepared AcChR membranes revealed orders of 300–370 Å spacing in the meridional direction, and in-plane near neighbor distances of 91 ± 1 Å (61). More recently, on alkali stripped membranes (11), we have obtained x-ray diffraction patterns which reveal up to 11 orders of a 294 ± 5.5 Å lattice spacing between stacked vesicle membrane pairs, and 90 Å nearest-neighbor distance in the plane of the membrane (Fig. 8 *A*). In toxin-treated samples, however, the lattice spacing in both meridional and equatorial

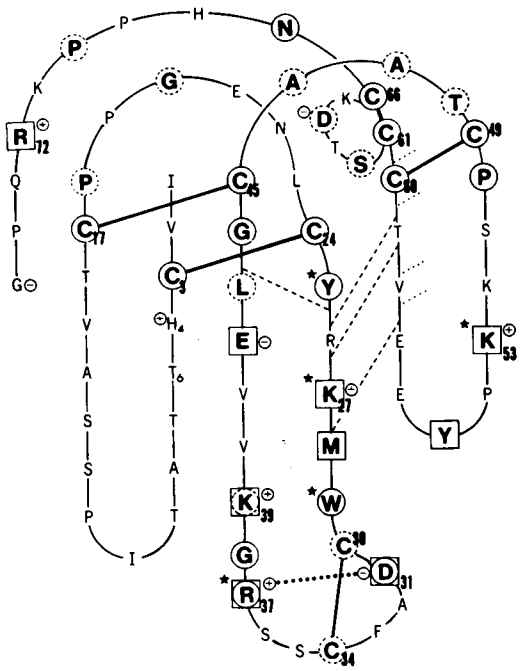

FIGURE 7 A schematic view of the amino acid sequence (50) and three loop structure (51) of α-bungarotoxin. Residues which are highly conserved among (*a*) all of the curaremimetic toxins, both "long" and "short" are enclosed in solid circles; (*b*) all of the "long" toxins but not between the long and the short are in dashed circles. Amino acids where chemical modification of the side chain has been shown to affect toxicity (53, 57) are indicated with a star; sites where an evolutionary change in the sequence seems to be correlated with a change in toxicity are in solid squares (53, 57). With exception of the four totally conserved cystine bridges, no single invariant or conserved residue has been shown to be essential for activity. It is therefore presumed that the residues contained within square boxes, and those marked with a star are in some way involved in the interface between α-bungarotoxin and AcChR, and all such side chains except R_{26} and W_{29} lie on the concave surface from which side the schematic is viewed. Dashed lines indicate hydrogen bonds between main chain atoms, and thick solid lines indicate the structurally important disulfide linkages.

directions changes, and ordering is diminished (Fig. 8 *B*). This observation suggests a toxin site which affects both in-plane neighbors and molecules in neighboring membranes, i.e., a site on the outside upper crest of AcChR (direct analysis of intensity (62) and electron density is in progress). (*b*) This interpretation is also consistent with our finding that membrane-bound AcChR preincubated with α-bungarotoxin is much more rapidly digested to the 27,000-mol wt limit digest pattern by trypsin (33) than AcChR in untreated membranes. (*c*) Antibodies raised against curaremimetic neurotoxins (i.e., most probably against toxin-host AcChR complex) have had specificities mapped onto the sequence, (63, 64) and hence into the structures of the toxins (56). Essentially all of the antigenic sites are located on the "back" convex surface which is not involved in the interface with AcChR. Furthermore, antitoxin antibodies bind to the toxin-AcChR complex (29). This implies that the toxin, and hence agonist, binding site is peripheral and not buried in the ionophoretic channel.

We conclude that the acetylcholine binding site is located within the toxin binding area, on the synaptic crest, and ~55 Å from the entrance to the most constricted region of the ionophoretic channel.

A FUNCTIONAL MODEL FOR AcChR

The large distance between ligand site and the entrance to the ion channel (which must be the gated channel), ~55 Å, raises questions as to how the binding of acetylcholine can lead to opening of an an ion channel. The mechanism could fall into either of two classes: the effect of acetylcholine could either be communicated to the channel by a detailed relay of coupled side chain motions, focusing the allosteric effect to a critical gate within the channel, or it could involve a large "global" change in conformation induced in the oligomer by ligand binding. Several factors strongly suggest the second kind of mechanism, that the conformation changes involved in channel opening, channel closing, and desensitization are large. Firstly, the reactivity of three out of four types of receptor subunits to photolabeling by bisazidoethidium bromide is altered by ligand binding (65). Secondly, antisera have been prepared which can inhibit channel opening without affecting ligand binding (66). Can they, like neurotoxins perhaps, "lock" the quaternary structure and therefore prevent the necessary large quaternary change? Thirdly, AcChR binds ~16 terbium ions of which half can be displaced by ligand binding (67). Again, the suggestion is for a large configurational change of the molecule. The channel itself must be large, at least 6.5 Å across, yet must present a large energy barrier to the passage of ions in the closed state. It is difficult to imagine how any small configurational changes, say swinging of a tyrosine side chain at the end of an allosteric relay, could regulate such large changes in the free energy barrier to ion permeability. Thus we contend that the cited evidence strongly supports a mechanism which involves a global change between the closed channel state and the ligand-induced open channel state.

We now propose a testable hypothesis for the mechanism of AcChR (see Fig. 9 and reference 68). We postulate that binding of agonist changes the quaternary state from one stable resting or closed state, via an open channel state, to a second stable, closed state that is quite different from the first, although it has essentially the same ground state free energy. This kind of mechanism can satisfyingly explain many properties of the ligand-induced channel opening events. We postulate that one subunit, α_1, is in a "primed" state (α_1'), different from that of the other (α_2). Ligand binding to both subunits together (Fig. 9 *i*), leads to channel opening with a rate constant k_1, represented in Fig. 9 by alignment of the extended α-subunits around the open channel (Fig. 9 *ii*). We postulate that the second

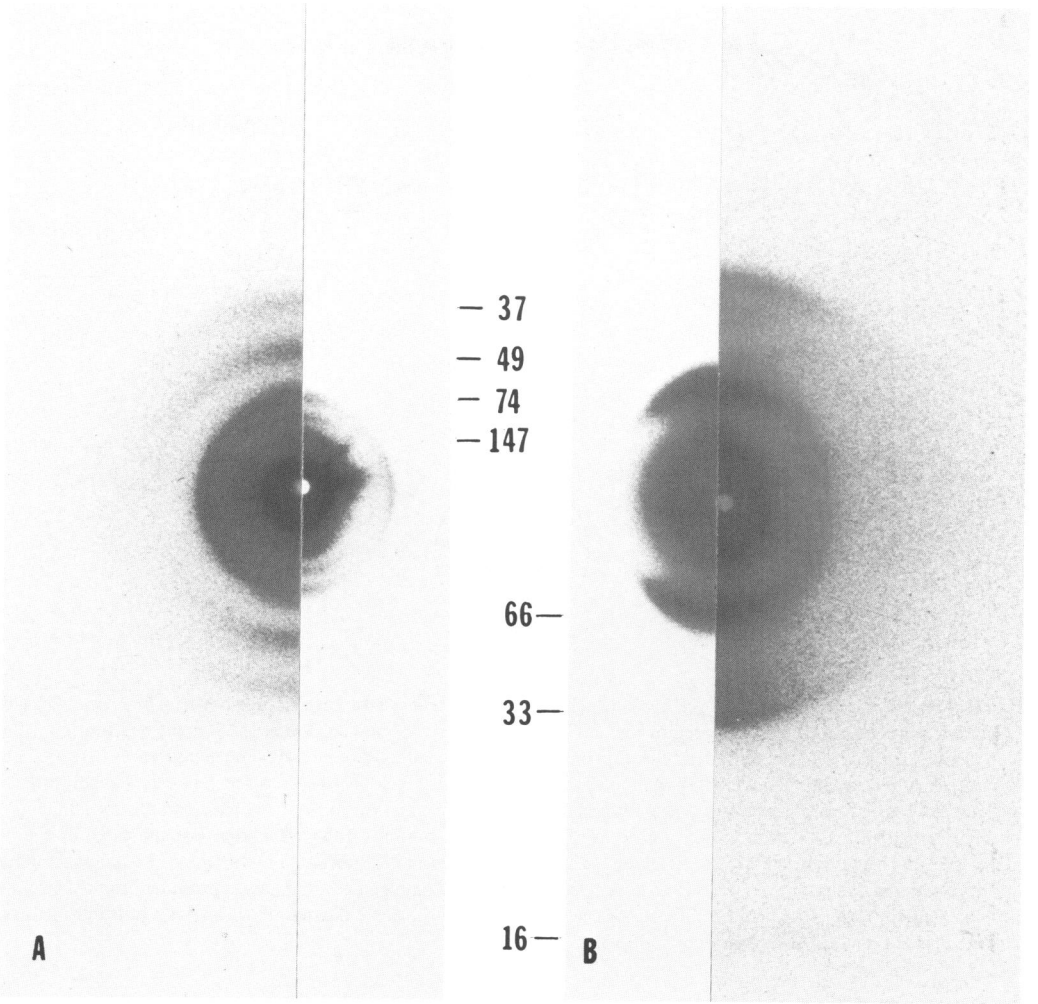

FIGURE 8 X-ray patterns recorded from alkali stripped membrane preparations. (*A*) Native membranes show 11 orders of an inter-vesicle repeat spacing of 294 ± 5.5 Å throughout the pellet. A shorter exposure is shown in the right half of the panel, and spacings in Å are indicated parallel to the upper meridion. Even diffraction orders are much stronger than odd orders; 1 = 2,4,6,8 are most obvious in the figure. (*B*) Membranes with α-bungarotoxin added show a dramatic change in both meridional and equatorial spacings and in the variation of these spacings within the pellet. Spacings below the center of the figure identify the main, correspondingly broader reflections along the meridian.

α-subunit (α_2) now reverts to a configuration similar to that initially held by α_1 and so closes the channel with a second rate constant k_2. This closed quaternary state (Fig. 9 *iii*) is different from (Fig. 9 *i*), but also a stable closed state. Acetylcholine can diffuse away from α_1 and α_2 without evoking a microscopically-reversible, channel-opening event. A concommitant relaxation allows the α_2 subunit to resume a configuration like that previously held by α_1, (Fig. 9 *iv*).

The next event would first lead to channel opening (Fig. 9 *v*), then ultimately change the quaternary state (Fig. 9 *vi*) back to the initial α_1', α_2 state (Fig. 9 *i*). In this mechanism the two possible open channel states are intermediate states whose lifetimes are determined primarily by the barrier to a change in the structure of agonist-bound AcChR itself, and are therefore independent of agonist concentration.

This proposal is a "cyclic" mechanism, and to close the cycle the channel is opened twice. It takes account of the evidence for a large conformational change in receptor and the inherent asymmetry of ligand binding at the 2 α-subunits. This proposal would also postulate that the asymmetric MBTA binding site should change upon channel opening from one subunit α_1', to the other, α_2'. It could explain how toxin or antisera binding can inhibit channel opening by locking a more distant (~55 Å from the channel) global quaternary structure change. The hypothesis also takes account of the fact that the channel does not reopen upon dissociation of ligand in the microscopic reverse of association. We present this idea as a means of focusing the wealth of data now emerging on AcChR structure. We also seek to define the respective subunit locations in the molecule, to quantitate the motion of subunit polypeptide chains, to define the ends of the 80 Å

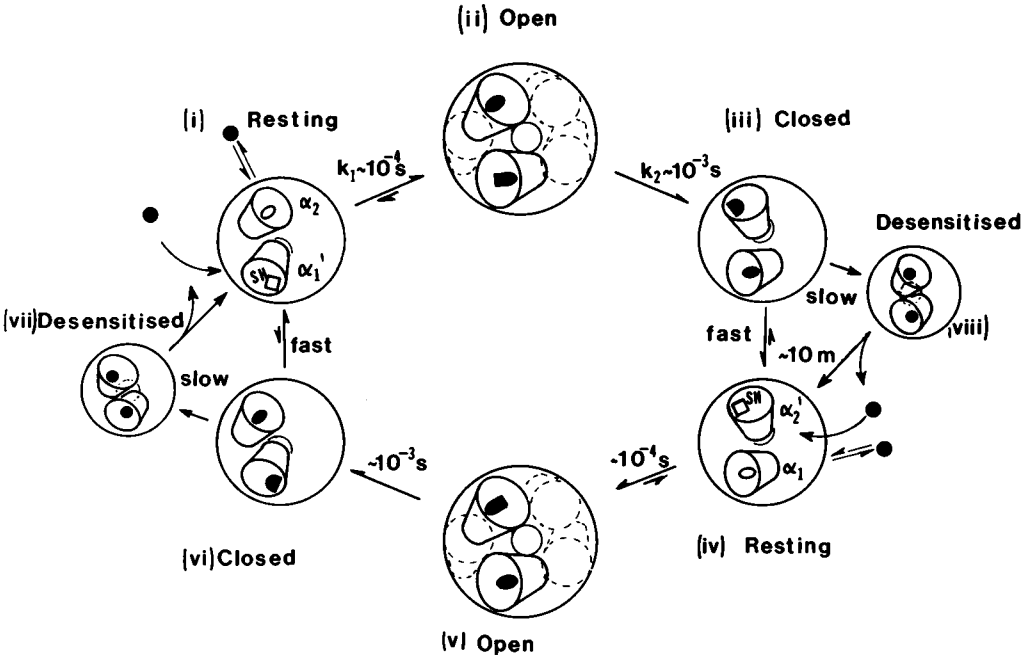

FIGURE 9 Proposal for a cyclic mechanism of AcChR in which a major quaternary structure change between one resting or closed state (*i*), and a second different resting state (*iii*), is triggered by agonist binding to both α subunits of which one is in primed configuration, and passes through an open channel state (*ii*). The open state is closed primarily by the second α_2-subunit (*iii*). The next event is triggered at the other α_2' subunit following a relaxation to a state in which α_2' resembles the state previously held by α_1 (*iv*). A second open channel state (*v*) is probably similar but not identical to the first, and it closes (*vi*) before relaxation to the original resting state which closes the cycle. Quaternary change is indicated purely schematically as motion of one or other of the elongated α-subunits. Slow relaxations are indicated as changing shape of the ligand binding site. In the desensitized states, (*vii, viii*) high affinity for ligand is represented by the most circular scheme for the agonist binding site. Arrows pick out the main pathways in the cyclic scheme. Other homologous subunits are presumed to pack around the central ion channel as indicated in (*ii* and *v*). Rough estimates of the rates (half-times) are indicated above major equilibria in the scheme. This "mechanical" scheme is not a minimal scheme consistent with kinetic data.[2,3]

helices in the sequence using chemical labeling techniques, and to determine the change in orientation within the AcChR induced by ligand binding. In collaboration with Dr. Jon Lindstrom and his colleagues we are attempting to decline binding sites for monoclonal antibodies in the AcChR, using tubular structures as described above.

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DISCUSSION

Session Chairman: Donald M. Engelman *Scribe*: Adam W. Dalziel

MCNAMEE: You said that toxin appears to destroy the lattice organization. Do other ligands also destroy the lattice structure? For example, it would be interesting to look at AChR with covalently-bound bromoacetylcholine.

STROUD: We don't know whether or not reagents such as bromoacetylcholine break up the lattice.

MCNAMEE: If a fairly large conformational change is associated with channel opening and desensitization, could you detect it?

STROUD: Yes, at 30-Å resolution we can still hope to detect changes of a much smaller distance. A difference Fourier transform map can detect changes of 6 Å at 30 Å resolution. In collaboration with Sebastian Doniach, Robert Fairclough, and Keith Hodgson, we have investigated conformational change of the receptor using anomolous x-ray scattering from Terbium ions. George Hess has showed that these ions bind to the receptor but are removed by the binding of ligand.

MCNAMEE: George Hess has presented a detailed kinetic scheme for receptor-mediated ion flux based on initial rate studies in eel vesicles (Hess et al. 1979. *Nature [Lond.]*. 282:329–331). Your model is more complex and more speculative. Have you tried to reconcile your model with Hess's?

STROUD: Fig. 9 shows our proposal. It is not a minimal model for the action of the receptor, but it was designed to deal with the question that if ligand binding to the receptor leads to channel opening (in agreement with the model of Hess, who has quantitated the rates of many of these steps), why does ligand diffusing away not lead to channel opening again in returning to the same starting structure? Channel opening is dependent on agonist type and concentration, while channel closing is independent of either. It therefore depends on the receptor complex. Our model is a cyclic scheme; half of this model would almost be equivalent to Hess's scheme. Mark McNamee and George Hess now have evidence for two steps in the desensitization of torpedo receptor (Walker et al. 1981. *Biochem. Biophys. Res. Commun.* 100:86–98). One step is slow and one is fast.

MCNAMEE: Alkali extraction removes the 43,000-dalton protein. It has been suggested that this protein is involved in receptor organization. Does the 43,000-dalton protein have any effect on the lattices you obtain?

KISTLER: The tubes with the crystalline AChR arrangement can only be obtained with preparations which have not been stripped with alkaline pH and so the 43,000-dalton protein is present in the preparations. However, we have not yet attempted to use anti-43K antibodies to determine if the 43K protein is present in the tubes. If it is, then we would presume that it is attached to the inside of the tubes since we know that the tubes are oriented with the synaptic side out.

STROUD: EPR studies by Philippe Devaux on the stripped and unstripped AChR membranes have been used to study the immobilization of the AChR. Devaux showed that the presence of the 43K protein leads to relative immobilization of the receptor.

EISENBERG: Why is it that the doublets in Fig. 3 are not seen in projection in Fig. 1?

STROUD: Only recently has it been possible to visualize individual dimers either by reconstitution (Sodel et al. 1980. *Eur. J. Biochem.* 110:13–33), by studies by Karlin and on co-workers isolated molecules (Karlin et al. 1980. *Cold Spring Harbor Symp. Quant. Biol.* 40:203–213), or by dispersing the AChR in the membrane by the technique described in our paper. Previously it was not possible to get molecules far enough apart to see individuals like that.

EISENBERG: In your paper you say that the apparently competitive binding among AChR, curare, and neurotoxins argues strongly that toxin and agonist-binding sites overlap. In fact, other (allosteric) mechanisms could be invoked to explain such behavior, and indeed you later invoke allosteric mechanisms themselves. But then, purely on the basis of evidence for toxin location, you state that you have located the AChR binding site. Aren't there gaps in the argument?

STROUD: Yes, there is the possibility that these sites do not overlap; however, very few people accept this. The binding is strictly competitive between these things: one will not bind while the other is there. Also the arganine aspartate ion pair in some toxins has been proposed (by Barbara Low) to mimic acetylcholine (Low et al. 1976. *Proc. Natl. Acad. Sci. U.S.A.* 73:2991–2994; Low et al. 1979. *Handbook of Experimental Psychology*. 52:213–257). In toxins which contain an asparagine instead of the aspartate, toxin binding is impaired. Thus we conclude that the binding sites overlap.

STEVENS: I am unconvinced by the claim in your paper's Discussion that densitometric measurements from micrographs of negatively stained specimens "unequivocally prove that stain penetrates throughout the entire transmembrane length of the receptor at a single central ion channel." This conclusion is based on several assumptions which are not individually or collectively plausible. In essence, you are assuming that the layer of stain and membrane material is uniformly 110-Å thick with the portion of stain being the complement of the membrane material. I can think of no reason of principle or precedent for believing this to be the case. A simple alternative explanation (and by no means the only alternative), is that your darker centers could be accounted for by some accumulation of stain above or below the rosette with relatively shallow stain between rosettes. Further, there are dark patches visible in parts of Fig. 3*b* which are not located at the centers of rosettes. Are these also ion channels?

This leads to another question: Do you have any further evidence to support the idea that it is possible to deduce a three-dimensional structure unambiguously from a single two-dimensional projection?

KISTLER: Everybody who works with negatively-stained specimens for electron microscopy knows that when you look over the grid at the whole specimen there is a lot of stain variation. Also, it is important which side of the specimen is in contact with the support film. In various cases (especially with periodic arrays) the near side of the film is best preserved and stained and has the most contrast.

With AChR membranes we are very fortunate. We adsorb vesicles to a carbon film which had been rendered hydrophilic by glow discharge. When the vesicles adsorb to the film they break open, especially with alkaline-extracted membranes. The vesicles are synaptic side out, and when they adsorb to the supporting film the side with the 50 Å protrusion is in contact with the support film. I am convinced that with a film made hydrophilic by glow-discharge you will fill in the space in between with stain. I acknowledge that this would not necessarily be the case if the situation were reversed and the membrane was attached to the grid by the cytoplasmic side. However, because we were fortunate in attaching the membranes on the synaptic side, the stain layer is as thick as 110 Å, or at least 100 Å if we give a little tolerance for the small 10 Å protrusion not being fully covered by stain on the cytoplasmic side. I am confident that the stain thickness is, in fact, 110 Å ± 10 Å.

STEVEN: I think that it is important to have something much more quantitative.

GAFFNEY: It seems that some of these questions could be answered by asking if any other cation's ligands compete with the uranyl stain. This might determine whether the uranyl is in the channel or not.

KISTLER: We have asked ourselves why the uranyl would enter the channels when we would expect them to be closed. The answer is we do not know.

STROUD: We do know that phosphotungstic acid does not apparently stain the channel so deeply. Also the electrophysiology has not been done with uranyl ions.

GAFFNEY: Do you expect to be able to get evidence from EM pictures for the proposal in Fig. 9 that an MBTA site can be located on either of the α-subunits, for instance by two types of alteration of a structure having particular handedness such as that shown in Fig. 5?

STROUD: This is something that Karlin and his colleagues have contributed to by using an avidin coupling through biotin to neurotoxins on the AChR in solubilized form. We have another approach to the question whether the MBTA site is always on one α-subunit rather than the other. It is known from Karlin and McNamee's work that one specific α-subunit binds MBTA while the other α-subunit does not (Karlin et al., 1975). McNamee has pointed out that the environment of each α subunit in this complex is different. If any of the binding sites were close to neighboring subunits they are intrinsically asymetric.

STEITZ: How might the AChR assemble so that one set of α-subunits would interact only with δ and β while the other would interact with β and γ? What prevents polymerization of different sorts?

STROUD: It has been shown that each molecule contains the same combination of subunits. The sequence analysis shows that all the subunits are homologous. We propose that these subunits are arranged like five staves of a barrel and that these stave-like interactions specify the general nature of the surrounding of the channel. Presumably some other determinants of the sequence specify the nearest neighbors. Once the assembly has started the system is asymetric. For example, an α-γ combination might have a unique structure which specifies its nearest neighbors.

WOLBER: Are the two α-subunits orientated vectorially the same way?

STROUD: Yes.