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# STRUCTURE AND CHARACTERIZATION OF THE ION-CHANNEL OF THE NICOTINIC ACETYLCHOLINE RECEPTOR

ANNUAL REPORT

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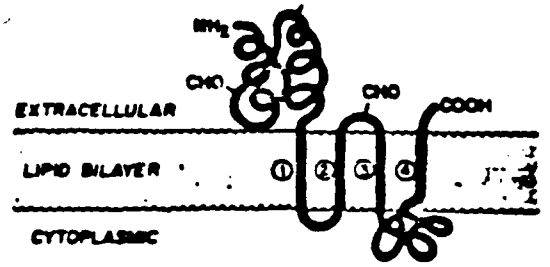
## Background

### 1. Receptor Structure

The nicotinic acetylcholine receptor (AcChR) is a complex of four polypeptides of molecular weight 40,000, 50,000, 60,000 and 65,000 daltons, generally referred to as α, β, γ, δ, which we first described (1) and this has since been confirmed in several laboratories (2). By performing amino-terminal amino acid sequence analysis of these four polypeptides we demonstrated that they are a highly homologous set of proteins (3) presumably derived from a single ancestral gene and further that they are associated with a stoichiometry of $\alpha_2$ β γ δ in AcChR from the electric ray, Torpedo californica, the most convenient source of this receptor molecule. Based on the amino acid sequence data we obtained, four groups constructed nucleotide probes for the isolation of C-DNA for subsequent sequence determination. The group of Barnard (4) was first to sequence the α-chain C-DNA of Torpedo AcChR, Patrick et al. sequenced the γ chain (5), Numa and his colleagues sequenced all four C-DNA molecules (6-8) and the α-chain sequence of Barnard has recently been confirmed by Changeux and his associates (9). Some of these groups have suggested a model of the segments of the subunits that span the postsynaptic lipid bilayer (5, 8, 9) based on recognition of stretches of hydrophobic amino acids in selected regions of the polypeptides (see Fig. 1). Other models that differ somewhat




Model depicting possible partitioning of the γ-subunit polypeptide chain in the lipid bilayer. Potential asparagine-linked glycosylation sites are marked with hatched CHO. The numbers 1–4 correspond to putative membrane-spanning regions.

Figure 1

have also been put forward (10-12). They are of course speculative and it is clear that a great amount of work needs to be done in order to specify the folding pattern of the receptor polypeptides and their relationship to the lipid bilayer. These studies are clearly relevant to the human or mammalian AcChR since we have recently shown that fetal calf AcChR is very closely related in its primary structure to that of Torpedo AcChR (13).

### 2. Cation Gating Function of the Receptor

Voltage noise analysis of muscle postsynaptic membranes (14-18) has allowed determination of the efficiency of monovalent cation transport by a single receptor channel which amounts to $10^7$ such ions per second. By using membrane vesicles isolated from Torpedo electric organ that contain no protein components other than the four receptor polypeptides, we were able to demonstrate that each receptor molecule could transport $6 \times 10^6$ monovalent cations per second (19). This was made possible by the development of a rapid stopped-flow spectroscopic method which is based on entrapment of a water-soluble fluorescent small molecule within the vesicles. The rate of fluorescence

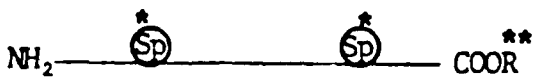
quenching by monovalent ions such as $Tl^+$ or $Cs^+$ by a heavy atom effect as the ions penetrate through acetylcholine activated channels in the membrane allowed the necessary quantitation. The major conclusion reached was that the AcChR responded in vitro in a manner identical to that found in vivo with respect to its transport efficiency and that therefore the recognition site(s) for the neurotransmitter and the channel for ion-transport are both intrinsic to the pentameric complex formed by the four subunits. Determination of the elements that form the channel is a major goal.

## Approach to the Problem

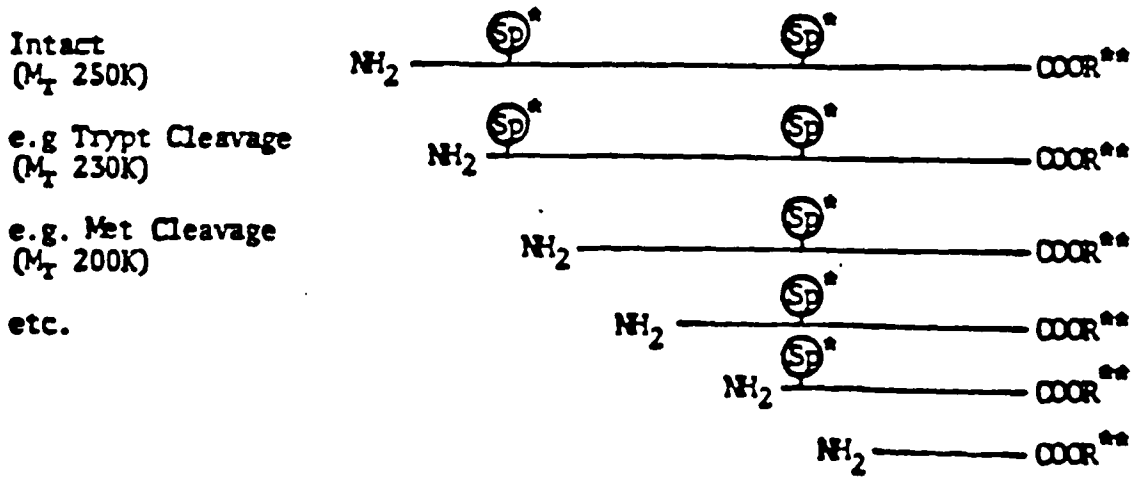
### 1. Membrane Topography

We utilize sealed, right-side out AcChR enriched membrane vesicles and vesicles that contain only the AcChR as protein components (20). We have previously shown (21, 22) that all four subunits are exposed to proteases on both sides of the membrane.

Our approach to determining the polypeptide folding pattern through the membrane is first to develop a method for specific labeling of the COOH terminal of protein molecules. Applied to the receptor system this would allow us to determine which side of the postsynaptic membrane each COOH terminal is on and substantiate or eliminate aspects of the simple folding patterns proposed from the C-DNA sequence work (5, 8-12). The second part of the approach necessitates a second labeling of the receptor subunits from inside or outside the vesicles. The object is to identify the regions labeled with chemicals or enzymes from either side of the bilayer or within the bilayer. As an example suppose we label the outside of the membrane with an enzyme such as transglutaminase and $^{14}C$-spermidine (* Sp) and that two regions of a given polypeptide are labeled as shown.




Degradation of this by a variety of enzymes or specific cleavage reagents will yield mixtures of polypeptides, some labeled with both [$^3H$] and $^{14}C$ and some with one or the other. Their identification is the key to the method, i.e.




This can be achieved by first identifying which Coomassie Blue stained bands on a high resolution (long with optimal acrylamide and crosslinker concentrations or gradients) gel for the polypeptide system are radiolabeled using fluorography. Cutting out these bands followed by digestion and counting for $^{14}C$ and [$^3H$] will allow identification of doubly labeled polypeptides as well as quantitation of the relative amounts of each

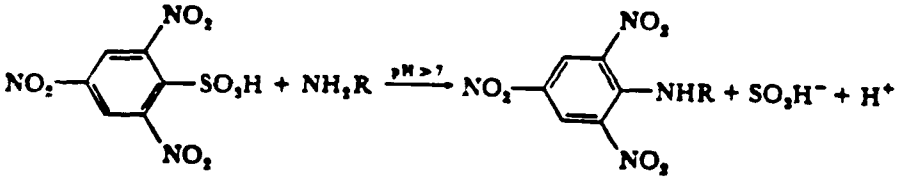
label. (Another approach is to label the protein separately, do parallel autoradiographs and compare visually—and confirm identity by microsequence analysis if necessary.

By conducting these studies with a variety of enzymes (e.g., transglutaminase, lactoperoxidase, protein kinase) or membrane impermeant chemical agents ($^{35}$[S] NAP-taurine, [$^{14}$C] isethionyl acetimadate) labeling from either outside or inside can be achieved. Labeling from within the bilayer can be accomplished by photolabeling procedures using organic soluble azides (23) or diazirines (24). By subjecting the system to a few such labeling procedures and identifying the segments of a defined set of polypeptides of decreasing $M_r$, it should be possible to generate a general folding pattern for the membrane protein.

Two other methods are also proposed to label specific aspects of the receptor subunits. The first of these is [$^{3}$H] adamantanediazirine which will be used to label those segments of the AcChR polypeptides that made contact with the lipid bilayer of the postsynaptic membrane. This reagent, originally devised by Bayley and Knowles (25) has significant advantages over more commonly used aromatic azides (25). Indeed, using such reagents conflicting reports on which subunits of the receptor contact the bilayer have come from Gitler's laboratory (26) where only the α-subunits were labeled and from Martinez-Carrion's laboratory (27) where the γ and δ subunits were labeled.

The second photolabile reagent is [$^{3}$H] diazoacetylcholesterol, the nonradiolabeled derivative having been recently described (28) as a reagent for labeling regions of membrane proteins that interact with the lipid-aqueous interface, thus allowing potential definition of specific folding patterns after identification, by sequence analysis, of the labeled amino acids.

A further approach which we are working on is to determine the location of the amino termini of the four receptor subunits with respect to the exterior and interior membrane surfaces. We use a membrane impermeant reagent for amino groups (both α- and ε- will react), namely trinitrobenzene sulfonic acid (TNBS) which is, as we have recently shown, impermeant and which forms α- and ε - trinitrophenyl derivatives.




Using sealed vesicles it should be possible to determine whether the α-amino groups are exposed by comparing results from such sealed vesicles with those obtained using vesicles rendered permeable to TNBS by freeze-thaw procedures (19). Identification of the α-terminal residues (known from our sequencing studies (3) will be achieved as follows: the labeled AcChR will be hydrolysed in acid to yield amino acids, α-TNP-terminal residues and ε-TNP-lysine residues. The α-TNP-terminals will be specifically extracted into an organic solvent such as dichloromethane from acidic solution of pH 2-3, concentrated to dryness and the α-TNP groups removed by treatment with ammonia (29). The terminal amino acid residues will then be quantitated using amino acid analysis.

2. Labeling and Identification of Putative Ion Channel Structures

The ideal method of labeling ion-channels would be with an ion as it passed through the open channel. We propose to develop such a labeling procedure. The AcChR channel is strongly preferential for cations and will allow many inorganic and small organic cations to pass through it in response to agonist binding (20, 30, 31). The channel, in addition, should stay open, in the absence of acetylcholinesterase, for a sufficient time for chemical reactions to occur, i.e., many milliseconds depending on the agonist concentration and therefore on the rate of desensitization (30).

The requirements for labeling by an ion are (i) that it be capable of being activated to a state from which it can chemically react with components of the channel, (ii) that a method is available for activating it in the channel, and (iii) that it can be obtained as a radioisotope. Chromium fulfills all these requirements. It is proposed to use $^{51}Cr^{++}$ (chromous) ion as the label. The protocol will be as follows: all experiments will be conducted in an Argon atmosphere to prevent oxidation to $Cr^{+++}$. The AcChR vesicles can be loaded with the $^{51}Cr^{++}$ by osmotic shock or by freeze-thaw cycles (19) and the exterior $^{51}Cr^{++}$ removed by gel filtration. The critical experiment will involve adding an agonist to elicit efflux of the $^{51}Cr^{++}$ through the open channel. In order to activate at least a fraction of the $^{51}Cr^{++}$ in the channel an oxidant will be added simultaneously with the agonist. This will be $Cu^{++}$ which should also diffuse through the channel in a direction opposite to the chromium and upon encounter cause outer sphere oxidation to $^{51}Cr^{+++}$ with generation of Cuprous ion. Since ligand exchange is rapid from $^{51}Cr^{++}$ but extremely slow from $Cr^{+++}$ it is hoped to effect binding of the $^{51}Cr^{+++}$ to ligands in the channel such as R-OH, R-COOH, etc. Crosslinking is also possible between two or more polypeptide chains. In the case of the AcChR SDS gel electrophoresis will be used to separate the four subunits and any crosslinked subunits. Pairs (or more) of crosslinked subunits will be readily identified by microsequence analysis. Such products, i.e., subunits or crosslinked subunits would be degraded to peptides and the radiolabeled spieces separated and microsequence analysis performed on them. Comparison with known sequences of the four receptor subunits would permit identification of exactly which parts of the AcChR were labeled.

3. Specific Tasks to be Performed

(a) Task 1: Localization of carboxyl(COOH) terminals. COOH terminals of acetylcholine receptor (AChR) subunits shall be labeled with [$^{3}$H]amino acids under conditions that promote a reversal of the usual catalytic activity of carboxypeptidase. COOH terminals shall be localized to the inside or outside of the membrane by addition of carboxypeptidase to the inside or outside of sealed, right-side out AChR enriched membrane vesicles and vesicles containing AChR subunits as the only protein components.

(b) Task 2: Determination of AChR polypeptide folding pattern through the membrane. COOH terminals shall be [$^{3}$H]-labeled as in section a, and other regions will be radiolabeled with chemicals or enzymes from either side of the membrane lipid bilayer or from within the bilayer. The radiolabeled AChR shall be degraded using enzymes or specific cleavage reagents. The resulting polypeptides will be separated by SDS-polyacrylamide gel electrophores Peptides containing one or both labels will be subjected to amino acid analysis and/or micro sequence analysis to determine the precise location(s) of the radiolabel in the primary amino acid sequence of each subunit.

(i) Labeling from outside or inside the membrane will be accomplished using a variety of enzymes (e.g., transglutaminase, lactoperoxidase, protein kinase) and membrane impermeant chemical reagents ([$^{35}$S]NAP-taurine, [$^{14}$C]isothionyl acetamidate).

(ii) [$^3$H]adamantane diazirine will be used to label AChR amino acids within the lipid bilayer. For these studies, the COOH terminal and lipophilic regions of the receptor will be labeled in separate preparations of AChR-containing membrane vesicles. After controlled degradation, peptides that are labeled in both preparations will be identified by visual comparison of parallel polyacrylamide gel autoradiographs.

(c) Task 3: Identification of ion channel components. $^{51}Cr^{2+}$ shall be used to covalently radiolabel ion channel structures during agonist activation of the AChR. AChR vesicles will be loaded with $^{51}Cr^{2+}$, and chromous ion outside the vesicles will be removed by gel filtration. To elicit efflux of $^{51}Cr^{2+}$ through the open channel, an agonist will be added to the preparation. An oxidant, e.g., $Cu^{2+}$, will be added with the agonist in order to generate $^{51}Cr^{3+}$ inside the ion channel. $^{51}Cr^{3+}$ should covalently bind to moieties in the channel, and may also cross-link polypeptide chains. Radiolabeled portions of the AChR will be identified and located within the primary amino acid sequence. These experiments shall be conducted under an inert atmosphere to prevent spontaneous oxidation of $Cr^{2+}$ to $Cr^{3+}$.

## Results

### 1. Carboxyl-Terminal Labeling of Proteins and of the Acetylcholine Receptor

The following discussion will be devoted to our studies for optimization of the carboxypeptidase Y catalyzed labeling of selected proteins with [$^3$H] labeled amino acids and amino acid derivatives, as well as with their non-radioactive counterparts. As examples glucagon, ribonuclease-S peptide and ribonuclease S protein will be discussed.

(a) Glucagon. This polypeptide is known to have little or no secondary structure and is therefore a good model to examine the possible side-reactions of carboxypeptidase Y catalyzed insertion of amino acids at the carboxyl-terminal. Using conditions previously utilized for synthesis of small peptides (32) by the enzyme at high pH (9.5), high enzyme concentration (mg/ml) and high nucleophile (amino acids, amino acid amides or other amine nucleophiles) concentration (0.1-0.5 M). Using 20 mg glucagon, 1 mg/ml carboxypeptidase and 0.1 M methionine amide we observed that considerable incorporation of the amino acid amide was obtained. Two products could be isolated, namely a precipitate which, upon extensive washing and dialysis to remove free methionine amide, yielded an amino acid composition consistent with the addition of an average of eight methionines to the COOH terminal and a soluble product, isolated by gel-filtration that had, on the average, two methionine residues added. Similar results were obtained in the presence of added acetamide and sodium acetate, added as scavengers to inhibit the reaction of the side chains of glutamine and asparagine, glutamic and aspartic acids, respectively. In these cases the number of methionine residues incorporated were about half of that observed in the absence of the scavengers. Amino terminal amino acid sequence analysis of the various samples showed clearly that in the absence of scavengers abnormal peaks were observed in the HPLC analysis of the PTH amino acids produced at positions where GLN, ASN, ASP, GLU residues were expected. In the presence of scavengers no such abnormal peaks were observed (Fig. 2).

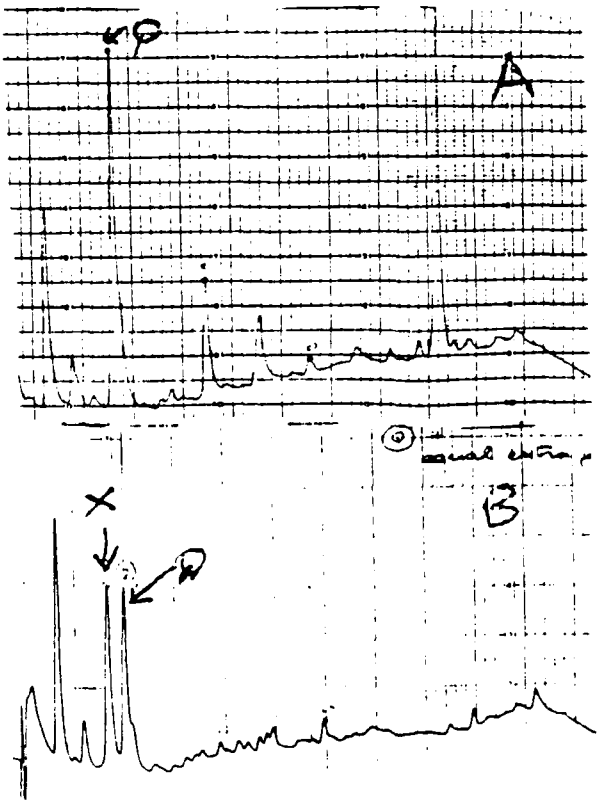



Figure 2. Separation of PTH amino acids with (A) and without (B) scavengers present. The expected residue is Q (glutamine). In the absence of scavengers a major extra peak of unknown structure (X) is present.

(b) Ribonuclease-S protein. Addition of methionine residues to this protein under the conditions described above, using methionine amide as nucleophile, yielded a precipitate that contained about 5 extra methionine residues upon amino acid analysis and a soluble product, isolated by gel filtration, that contained on the average about 4 additional methionines. Amino-terminal sequence analysis showed that in this case no abnormal peaks occurred in the HPLC analysis of PTH amino acids at expected GLN, ASN, GLU or ASP positions. This result suggests that proteins with extensive secondary and tertiary structures are not subject to side-reactions at these residues as was shown for glucagon (above) where such structure was lacking. Therefore side-reactions appear to be much less of a problem with native proteins having extensive three-dimensional structure.

(c) Ribonuclease-S-peptide. In contrast to the two examples discussed above no incorporation of methionine residues occurred using carboxypeptidase Y at pH 9.5 and methionine amide as nucleophile. This was at first puzzling but upon discovery that the COOH terminal sequence of this peptide strongly resembles cytochrome c- (Tuna)

(i) S-peptide Ser-Thr-Ser-Ala-Ala-COOH

(ii) cyte Lys-Ser-Ala-Thr-Ser-COOH

which is relatively insensitive to carboxypeptidase Y degradation (33) the result could be understood. Amino acid analysis showed that the terminal alanines were lost during

the enzyme treatment. These data lend further support to the notion that carboxypeptidase Y catalyzed incorporation at pH 9.5 of amine nucleophiles into proteins is at the COOH terminus.

(d) Acetylcholine receptor. The AcChR, purified by affinity chromatography was incubated with carboxypeptidase Y (1 mg/ml), [$^3$H] methionine amide (0.1 M) at pH 9.5 in carbonate-bicarbonate buffer (0.5 M). Following reaction for one hour the products were separated by gel-filtration (Fig. 3), demonstrating that the AcChR and

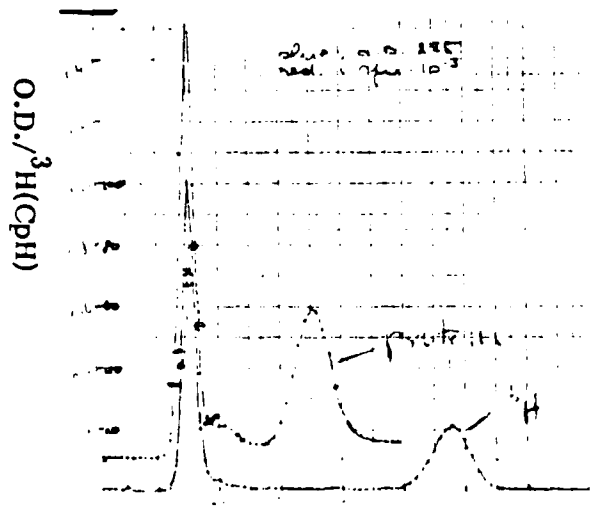



Figure 3. Gel-filtration profile (p-200 BioRad) of AcChR labeled with [$^3$H] methionine amide. The central peak is carboxypeptidase Y and the last peak is free [$^3$H] methionine amide. The amount of which was drastically reduced by dialysis prior to running on the column.

carboxypeptidase Y were both labeled and were each separated from the free amino acid. In addition SDS-gel electrophoresis of the labeled receptor demonstrated that all four subunits of the receptor were labeled as revealed by fluorography (Fig. 4).

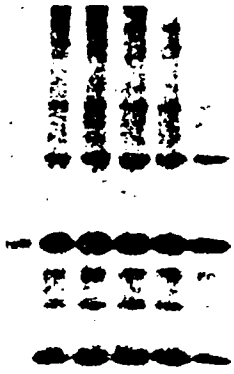


Figure 4. Fluorogram of SDS gel electrophoresis separation of carboxyl terminal labeled receptor subunits.

2. Polypeptide Folding Pattern through the Membrane

(a) Carboxypeptidase Y digestion of COOH termini in intact vesicle preparations. As a preliminary to labeling COOH termini by reversing the carboxypeptidase reaction at high pH by driving the reaction in reverse we first treated intact vesicles with the enzyme using conditions normally used to liberate COOH-terminal amino acids at neutral pH. The rationale for this resides in the following facts: we first demonstrated (34) that in addition to the pentameric form of the AcChR ($M_r$ 270,000) which sediments as a 9 S particle the AcChR also occurs as a 13.7 S form which others and we also showed to be due to one or more specific disulfide bonds between the δ subunits (x, y, z, a). Following the C-DNA sequencing of all four subunits Numa and his colleagues suggested that such a disulfide most likely was formed by cysteine residues of two delta chains near the COOH termini. These residues are the penultimate ones at the COOH terminus i.e.

$$NH_2(- - -)\text{-Cys-Ala-COOH}$$

and do not occur in the α, β or γ chains. Therefore it was possible that carboxypeptidase Y could dissociate AcChR dimers (13.7 S) into monomers 9 S in a specific manner. The results of such an experiment are shown in Fig. 5 where it is clear that the amount of 13.7 S dimer is decreased and correspondingly the amount of 9 S monomer increased with respect to control samples upon treatment with the enzyme. Since these experiments were conducted using sealed vesicles this result suggests that the COOH termini of the δ chains are on the outside surface of the membrane, in agreement with some models (6-8) (see Fig. 1) and in disagreement others (10, 12) regarding the localization of the COOH terminus. This result is also in disagreement with a recently published report (35) of the localization of the COOH terminus.

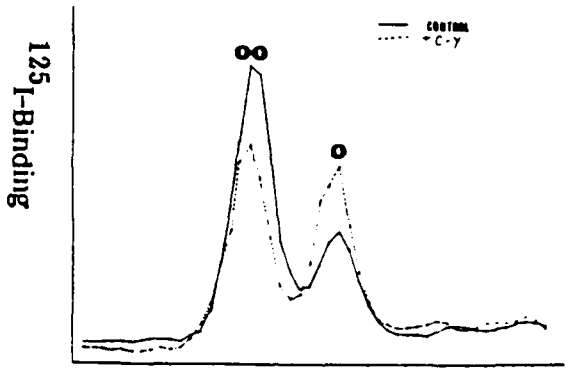



Figure 5. Density gradient separation of AcChR dimers (-) and monomers (O) without (-) and with (- - -) carboxypeptidase Y.

At present we do not have the instrumental capability to analyze for the amino acids liberated by carboxypeptidase Y but will if our request for a sensitive amino acid analyzer is approved by the U.S. Army Medical Research and Development Command.

(b) COOH-terminal labeling of the AcChR in sealed vesicles. The definitive answer, we believe, will soon be obtained by labeling the COOH termini of the AcChR subunits as outlined in our proposal, where we can isolate and sequence the radiolabeled COOH terminal peptides and we are now in the process of performing these experiments. We experienced a few months delay in doing this work due to the necessity to synthesize a new labeled amino acid, namely [$^3$H$_3$-methionine. The need for this reagent is that it is membrane impermeant, being a zwitterion whereas [$^3$H$_3$-methionine amide is permeant, being an amine. For general labeling of protein COOH termini the amide is preferable since incorporated yields are higher, whereas for membrane studies the amino acid is preferred. We now have the labeled amino acid in hand.

(c) Localization of the amino terminals of the AcChR in sealed vesicles. As outlined in the approach section we propose to locate the amino terminals of each of the four AcChR subunits with respect to the membrane using a membrane-impermeant, amino group selective, reagent to form trinitrophenyl amino acids under conditions of reaction outside and within sealed vesicles. This method can also be used to test some of the proposed polypeptide folding models by comparing the extent of lysine ε-group modification on the outside and inside surfaces. This can be determined spectrophotometrically since the number of lysines in each subunit far outweighs the single amino terminal. The extent of labeling can be compared by spectrophotometric comparison of identical aliquots taken from the two sets of labeling conditions. The factor we have determined is is 1.13, considering outside plus inside labeling versus outside labeling alone. Since the complete primary sequences of all four subunits are known the expected ratios of lysine residues outside and inside plus outside can be determined for the proposed models. In the case of the model shown in Fig. 1 (6-8) this ratio should be 1.7 while that proposed by others (10-12) would yield a ratio of 1.3. Thus our preliminary result favors the latter type of model, while nevertheless being considerably different from the proposed value. This emphasizes the necessity to keep in mind that the models proposed so far are probably great simplifications of the true structure and emphasize the need for extensive work to determine this structure.

(d) Interaction of the receptor with the membrane. It is accepted that the receptor complex spans the postsynaptic membrane although there is yet no definitive structure for how this is accomplished. Despite the wealth of information that has been accumulated on the receptor and membrane proteins in general, its interaction with the lipid bilayer and the functional role of membrane components still remains poorly understood. The lipid composition of electroplax membranes has been described (36-38). These membranes have a protein to lipid ratio of about 2.3 with the most distinguishing feature being a relatively high concentration of cholesterol (the molar ratio to phospholipid being about 0.9). This latter point appears to be a general property of nervous tissue and plasma membranes in general (39, 40).

One method of approaching this problem is to conduct reconstitution experiments. Such experiments suffer, however, from two major limitations. First, exposure to detergents and delipidation may result in denaturation or modification of the receptor (41-43) or in variability in properties of the final product. Secondly, in the investigation of the role of various lipids, it is difficult to distinguish between the requirement of a particular lipid for a successful reconstitution or its direct involvement in the function of the membrane protein. To avoid these limitations, we have developed a method of altering the membrane composition without removing the receptor from the lipid bilayer. AcChR-rich vesicles are fused with an excess of defined lysosomes using polyethylene glycol as the fusogen. This, in effect, dilutes out the native lipids and allows investigation of receptor function in a particular membrane environment. Because of the high level of cholesterol in AcChR membranes and indications that it may play an important role in receptor function, we have used this procedure to alter the level of the steroid in receptor vesicles. In addition, we have examined the effect of phospholipid composition on the flux rate.

Polyethylene glycol was used to fuse AcChR-rich membranes with vesicles prepared from lipids extracted from electroplax membranes. 30% PEG (w:w) was found to be sufficient to induce fusion. The extent of fusion was determined by equilibrium density centrifugation in 10-40% sucrose gradients. AcChR membranes have a very high protein to lipid ratio, therefore they are dense and band at the bottom of the gradient. On the other hand, phospholipid vesicles which contain no protein have a lighter density and float at the top. Introducing exogenous lipid into AcChR membranes should decrease their density and AcChR vesicles which have undergone fusion are expected to band at an intermediate density in the middle of the gradient.

In Figure 6 (A) sucrose gradient elution profiles of an unfused mixture of AcChR membranes and Torpedo lipid vesicles spiked with [$^{14}$C]-PC are shown. Duplicate tubes were run, one being trace labeled with [$^{125}$I]-$\alpha$ BuTx to detect receptor enriched membranes. As expected, the phospholipid vesicles remained at the top while the AcChR membranes sedimented to the bottom. The small amount of toxin binding at the top probably represents unbound or degraded toxin. In (B) duplicate samples of sucrose gradient profiles of the same mixture following fusion by 30% PEG are shown. Clearly, all of the AcChR membrane vesicles became less dense, indicating that they underwent fusion. The [$^{14}$C]-labeled lipid vesicles were split into two peaks, one remaining at the top and the other coinciding with the AcChR membranes. The coincidence of the receptor and [$^{14}$C]-lipid peaks indicates that the AcChR membranes incorporated exogenous lipid. The [$^{14}$C] peak remaining at the top represents homologous fusion events which result in no change in the vesicles density. Comparing the radioactivity under the two peaks reveals that approximately 50% of the lipid vesicles had fused with AcChR membranes. The relative sharpness and symmetry of the receptor vesicle peak indicates that the fusion procedure produced a rather homogenous population of vesicles as opposed to a heterogeneous distribution with a broad range of protein to lipid ratios.

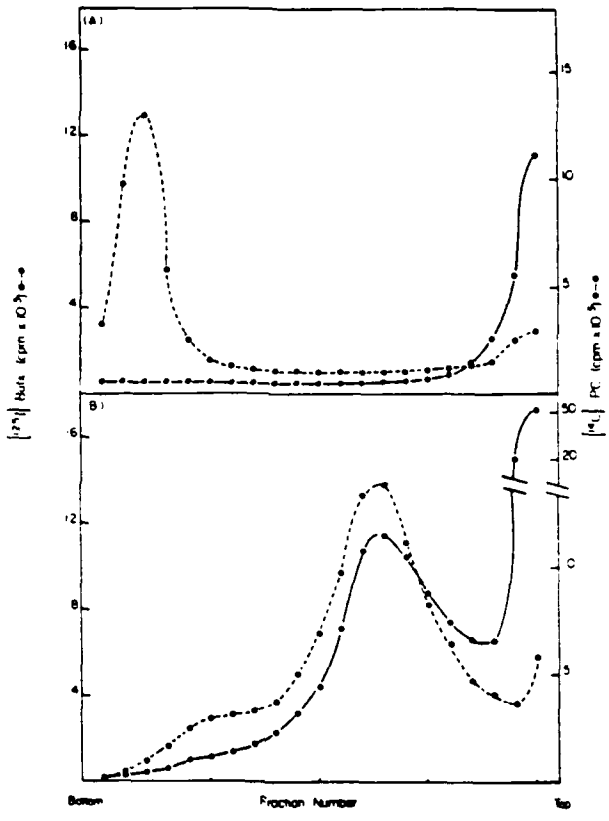


Figure 6. Sucrose gradient (10-40%) profiles of native and fused AcChR membranes. (A) AcChR membranes trace labeled with [$^{125}$I]-BuTx were mixed with lipid vesicles spiked with [$^{14}$C]-PC and applied to the gradient. (B) Same as in (A) except that the vesicle mixture was fused with PEG. The tubes were eluted by puncturing the bottom and fractions were counted for [$^{125}$I] or [$^{14}$C].

In Figure 7 electron micrographs of AcChR rich vesicles from Torpedo before and after fusion are depicted. The first panel (A) shows the extremely high density to which the receptor is packed in the purified electroplax membranes. The remaining pictures demonstrate the effect of introducing excess lipid into these vesicles. Clearly, the receptor particles have been significantly diluted and presumably now reside in a lipid environment more characteristic of the exogenously added lipid vesicles.

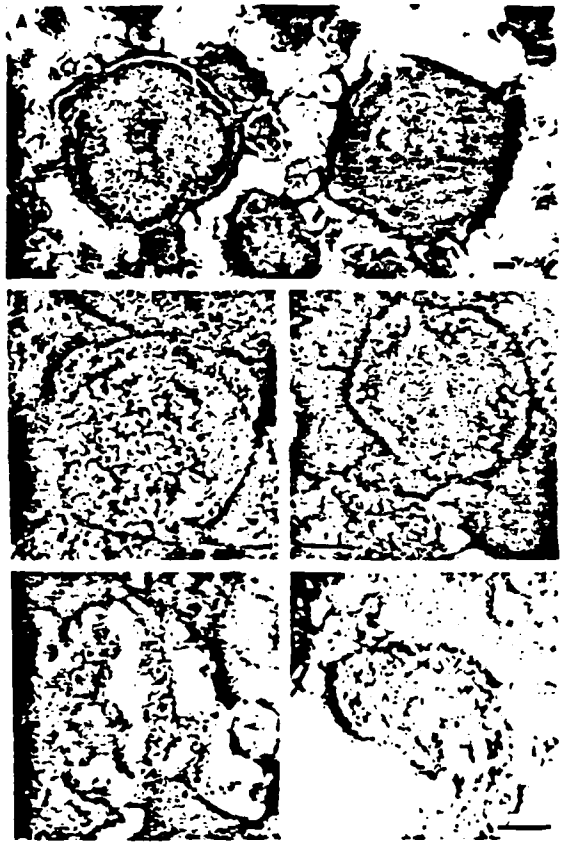


Figure 7. Electron micrographs of native and fused AcChR membranes. (A) Native membrane vesicles showing high receptor density. (B-E) Membranes following fusion with lipid vesicles. Preparations were negatively stained with uranyl acetate (bar = 50 nm).

Four lipid vesicle fractions were prepared using lipids from Torpedo electroplax. These contained the following proportions of cholesterol relative to that of native AcChR-rich membranes: 1, 0.5, 0.25 and 0. AcChR-rich membranes were fused with an excess of each lipid vesicle fraction. Taking into account the average lipid concentration of the AcChR membranes, typically a seven-fold excess of lipid vesicles was added. Assuming a fusion efficiency of about 50%, the final cholesterol levels in the AcChR containing vesicles were approximately those indicated in Figure 8. Since natural Torpedo lipids were used, the complement of phospholipids remained unchanged.

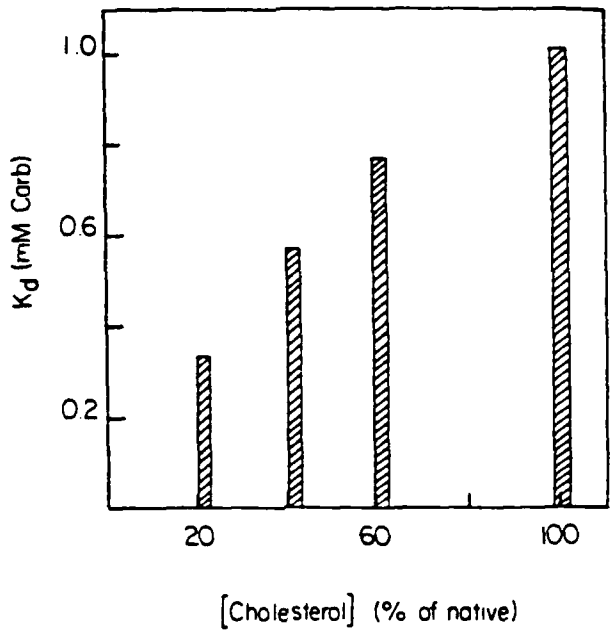



Figure 8. Histogram of $K_d$ for carbamylcholine versus cholesterol content of membranes as percentage of native level (values from Table 1). Cholesterol level was estimated by considering the excess of lipid vesicles added and the efficiency of fusion.

The AcChR vesicles with altered cholesterol content were then analyzed for carbamylcholine-induced $Tl^+$ flux. Fusion had the effect of lowering the receptor density in the membranes, i.e., reducing the number of receptors per vesicle and this produced slower apparent order rates of $Tl^+$ flux. The extent to which the receptor was diluted in these experiments resulted in flux rates in a workable range ($k_{max}$ + 50-80 $sec^{-1}$) avoiding the need to partially inactivate the receptor with HTX (19).

The results of carbamylcholine titration of the four vesicle preparations are given in Table 1. Comparison of the titration curves for "whole and cholesterol" and "X-lipid" fusions is shown in Figure 9 (see Experimental section for definitions). The values for the dissociation constants ($K_d$), determined by fitting the data to a binding isotherm, are averages of three separate experiments. Figure 8 is a histogram of the apparent $K_d$'s versus the approximate final level of cholesterol in the vesicle. The $K_d$ for carb consistently decreased (binding affinity increases) as the cholesterol level was reduced. Also, it was found that the saturating rate ($k_{max}$) decreased or remained the same with decreasing cholesterol. Considering that the average size of cholesterol depleted vesicles is smaller, as determined by electron microscopy, this means that ion flux is inhibited as a result of reduced cholesterol. (Smaller vesicles have less internal volume and thus would equilibrate faster, resulting in faster apparent rates.)

TABLE 1. Effect of Cholesterol on $K_d$[a]

| | Extracted | Whole + Extracted | Whole | Whole + Cholesterol |
|---|---|---|---|---|
| Cholesterol content relative to natural AcChR vesicles | 0 | .25 | .5 | 1 |
| | | $K_d$ (mM Carb) | | |
| $Tl^+$ flux[b] | 0.33 ± .06 | 0.57 ± .11 | 0.77 ± .18 | 1.02 ± .06 |
| NBD-fluorescence[c] | 0.38 ± 0.07 | - | - | 1.10 ± .14 |

[a]AcChR membranes were fused with an excess of Torpedo lipid vesicles which contained the indicated amount of cholesterol.

[b]Data fit to binding isotherm assuming two binding sites.

[c]Data fit to binding isotherm assuming one binding site.

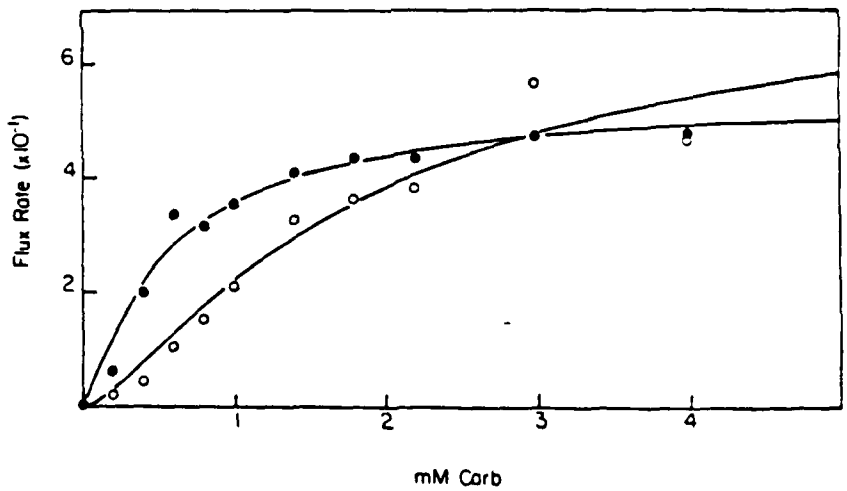



Figure 9. Dependence of the observed flux rate on carbamylcholine concentration. AcChR membranes were fused with "whole lipid" (O-O) or "X-lipid" (O-O) vesicles and assayed for carb-induced $Tl^+$ flux. The data were fitted to binding isotherm by assuming two ligands bound per activated channel.

In order to confirm the effect of cholesterol on the binding of agonist, AcChR membranes were labeled with the fluorescent ligand IANBD (4-[N-(iodoacetoxy)ethyl-N-methyl]amino-7-nitrobenz-2-oxa-1,3-diazole). These membranes were then fused with "whole plus cholesterol" of "X-lipid" vesicles as before. Titration with carb and fitting the data, assuming one ligand binding site, gave the values for $K_d$ listed in Table 1. Again, decreasing cholesterol was found to lower the dissociation constant.

The membrane fusion procedure was also used to modify the phospholipid composition of the AcChR membranes. Receptor membranes were fused with an 8-10 fold excess of vesicles prepared with PC and either PE or PS in the following proportions: 0, 0.1, 0.25, 0.5. Another sample was fused with vesicles which contained PS, PE and PC in the ratio 1:2:2. All the synthetic vesicles also contained 20% cholesterol (w:w).

The function of the receptor in these modified membranes was again examined with the $Tl^+$ flux assay. The $K_d$ for carb did appear to vary significantly in the different preparations but there was a change in the $k_{max}$. It is important to point out that by EM examination, the size distribution of the vesicles did not change with the phospholipid composition. Therefore, it was possible to directly compare $k_{max}$ and relate these values to the ability of the receptor to flux ions in the different membrane environments. Plots of $k_{max}$ versus phospholipid composition are presented in Figure 10, $k_{max}$ being determined at a saturating level of acetylcholine (1 mM). The flux rate increased as the mole percent of PE relative to PC increased from 0 to .5. For the PS/PC vesicles, the flux rate went through a maximum and then decreased to virtually no detectable flux at 0.5 mole percent. The bar graph at the right of Figure 10 represents the maximum flux in PS/PE/PC vesicles.

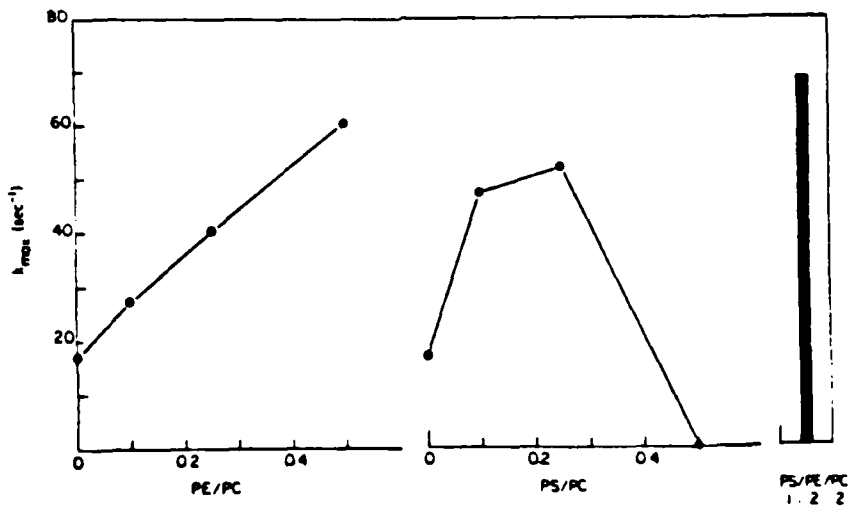



Figure 10. Maximum flux rate as a function of phospholipid composition. AcChR membranes were fused with an excess of phospholipid vesicles of the indicated composision. $k_{max}$ was determined as the apparent rate of $Tl^+$ flux at saturating levels of acetylcholine (1 mM). At the right is a bar graph of the maximum flux rate obtained following fusion with vesicles of similar composition to the native membranes.

In summary these results show that depletion of cholesterol from AcChR membranes increases the affinity of the receptor for agonist binding but at the same time decreases ion flux. The mechanism of how cholesterol might affect the functional properties of the receptor must still be determined. There are at least two possibilities: cholesterol may influence the bulk physical properties of the membrane to establish the proper membrane environment necessary for receptor function; alternatively, specific binding sites for cholesterol may exist on the protein surface (see below) which are responsible for modulating activity. The unusually high level of cholesterol in AcChR membranes would seem to favor the former model unless a series of low affinity binding sites exist for this lipid.

These experiments also indicate that the correct lipid environment is essential for receptor function. Much additional work will be required before the interaction of lipids with membrane proteins and their role in regulation is more clearly understood.

(e) Chemical labeling of the receptor from the bilayer. Previously we have reported that we have been able to demonstrate (24) that all four receptor subunits interact with the bilayer since all could be photolabeled with [$^3$H] adamantane diazirine as shown in Figure 11.

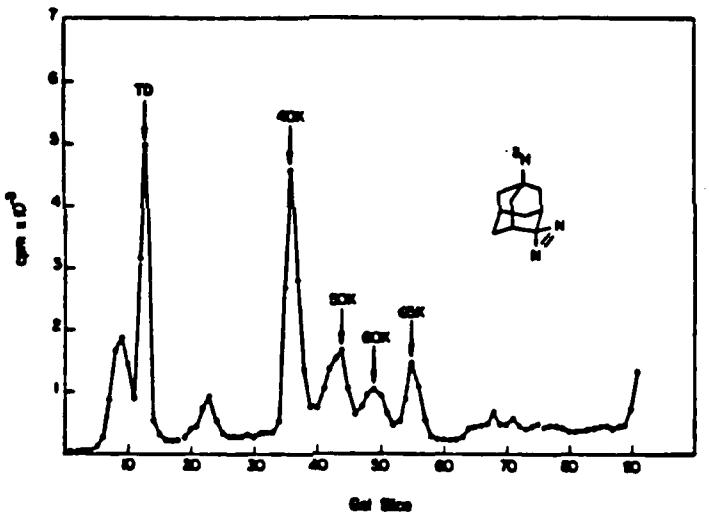


Figure 11. SDS gel pattern of [$^3$H] counts incorporated into AcChR subunits photolysed in the presence of [$^3$H] adamantane diazirine.

During the last six months of the granting period we have adopted a further approach to achieve a more specific and informative labeling from the bilayer. For this purpose we synthesized [7(n)-$^3$H] cholesterol diazoacetate. This was adopted for two reasons: (1) we wished to attempt to identify the subunit(s) that contain(s) the binding site(s) for cholesterol that affect(s) function of the receptor, as discussed at length above, (2) such an approach should allow labeling of the receptor at the water-membrane interface and thus facilitate identification of those precise regions of the receptor.

[7(n)-$^3$H] cholesterol diazoacetate dissolved in ethanol was introduced into membrane fragments enriched in AcChR. Exposure to UV light for 10 min, which was adequate for virtually complete photolysis, resulted in covalent labeling of all four subunits. As the probe cholesterol diazoacetate incorporates into bialyers in an fashion analogous to cholesterol this result confirms that the AcChR does interact with cholesterol.

In addition it suggests that there may well be binding sites for cholesterol on each of the subunits, not too surprisingly in view of the extensive homology between them, and it confirms that the desired labeling of the receptor at the water-membrane interface can be accomplished.

3. Identification of Ion-Channel Components

Labeling of the ion-channel of the AcChR has been continued, although we have had difficulty because of lack of proper equipment. Maintenance of chromous ion for any length of time has been the main problem since it is extremely susceptible to air oxidation to chromic solutions. Our only equipment for doing this as present are glove bags which are inadequate since it is impossible to prevent some leaks. We have now obtained the necessary monies (about $4,000) from Caltech to purchase an enclosure large enough and with specifications sufficiently good to exclude oxygen. This is on order.

So far we have attempted several experiments to label the AcChR with $^{51}Cr^{++}$ in the absence and presence of the agonist carbamylcholine. The preliminary results shown in Figure 12 are representative of many attempts to incorporate the label.

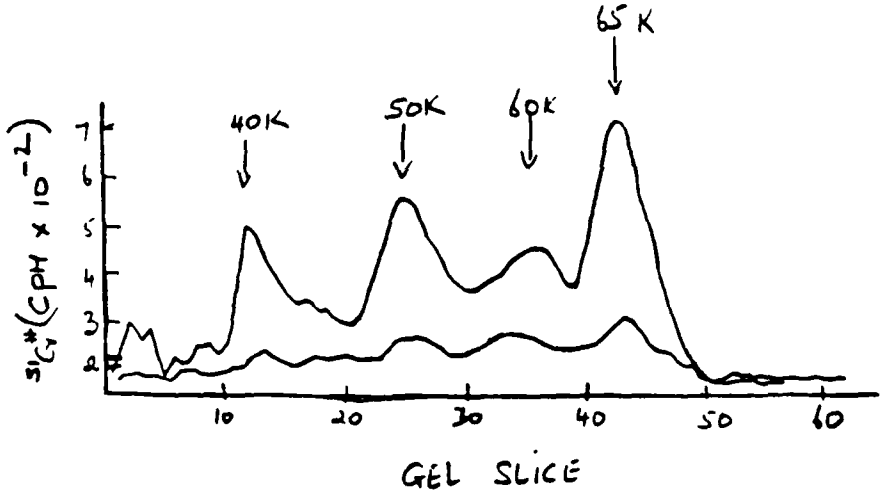



Figure 12. SDS gel electrophoresis of $^{51}Cr^{++}$ labeled AcChR in the presence (upper trace) and absence of added carbamylcholine.

It is seen that some labeling of all four subunits occurs in the absence of the ligand but that in its presence all four polypeptides are labeled to a greater extent (by a factor of about 5). The oxidant utilized was oxygen, injected with buffer or with buffer plus ligand. As mentioned in previous reports we expect cupric ion, added on the side of the membrane vesicles opposite to that from which $Cr^{++}$ will flow through the ion channel, to be a superior oxidant. These experiments are in progress but we do not expect rapid progress until we are in possession of the new controlled atmosphere enclosure for conducting the experiments under best conditions.

4. Improvements in Methodology

(a) AcChR vesicle preparations. We have improved our original preparation (44) of AcChR enriched vesicles over the years by various modifications (20). For much of the work being conducted in the studies described and anticipated here the most crucial aspects are (i) a highly purified preparation with essentially no protein components other than the AcChR, (ii) that all the vesicles be sealed. We have added an additional rapid centrifugation step following vesicle isolation (45). This allows selection of sealed over broken AcChR vesicles. The gradient used is 8-20% sucrose. Following reaction with the various reagents described in this report to select between outside and inside membrane surfaces the vesicles are again centrifuged to select against any vesicles broken during the performance of the experiment.

(b) Degassing of vesicle preparations. Degassing of buffers is a simple procedure and will present no problems once we have the controlled atmosphere enclosure in operation. Elimination of oxygen from vesicle preparations poses a more difficult problem. We have decided to adopt a simple approach to overcome this. It consists of passing the vesicle preparation down a G-25 Sephadex column as described previously (19) except that the column will be run in the controlled atmosphere enclosure using deaerated buffers and column resin. Exchange of gases and water is rapid through membranes and will result in the vesicle enclosed $O_2$ being trapped on the G-25 column by a gel-filtration effect.

5. General Structural Features of Acetylcholine Receptors

Due to the general interest in central nervous system receptors and their differing pharmacology and toxicology from peripheral receptors we have begun to investigate central receptors. The data reported here are the first of their kind.

(a) Purified optic lobe and brain AchoR. The method used for chick optic lobe brain AcChoR purification was a modification of a previous method (46) and was designed to minimize AcChoR proteolysis. These modifications were (i) solubilization for 1 hr in 1% Triton X-100 instead of 5% Lubrol PX for 2 hr, (ii) increase in the PMSF concentration 10-fold to 1 mM and the EDTA 5-fold to 5 mM, (iii) reduction of the period allowed for binding of AChoR to toxin-Sepharose from 18 hr to 3 hr, (iv) omission of the lentil-lectin-Sepharose treatment and of the recycling previously used during the elution by carbachol, and (v) replacement of the $^{125}$I-iodination used previously for detection in gels where necessary by silver or Coomassie staining, since lengthy iodination procedures allow proteolytic nicking of the subunits. The combined modifications led to some differences in the subunit pattern and sedimentation behavior from those previously described (46).

The purified α-BTX binding component from optic lobe showed high-affinity binding of [$^{125}$I-α-BTX with specific activities of 4000-6000 nmoles/g protein in different preparations. In sucrose density gradients it sedimented mostly as a peak with a sedimentation coefficient of 10.2 S. The component purified from brain had similar α-BTX binding characteristics and specific activity.

(b) Peptide composition of purified CNS AcChoR. Upon $NaDodSO_4$ gel electrophoresis the α-BTX binding proteins purified either from chick optic lobe or brain had very similar peptide compositions. Different preparations consistently contained 4-5 major components, whose molecular weights ranged between 48K and 72K. The component of lightest $M_r$ (48K) sometimes migrated in a diffuse form, which was poorly stained by Coomassie blue. This component was also very sensitive to proteolytic degradation, as demonstrated by its progressive disappearance with more lengthy purification procedures and also

during iodination. Similar patterns were observed when the specifically bound protein was either stripped from the toxin-Sepharose by $NaDodSO_4$ or specifically eluted by carbachol.

(c) Amino-terminal amino acid sequencing. All the peptides contained in purified optic lobe AcChoR were isolated and submitted to amino-terminal amino acid sequencing. The lowest $M_r$ component gave a readily identifiable single sequence. Comparison of this sequence with the known amino-terminal sequences of Torpedo, Electrophorus and calf peripheral AcChoR subunits, the α-subunit of chick and human muscle AcChoR (47-51) and the other subunits of chick muscle AcChoR (unpublished observations) revealed that the optic lobe sequence is, although different, highly homologous to the subunits of the other AcChoRs, the highest degree of homology being with the α-subunits and among these with the α-subunit of Torpedo (see Fig. 13 and Table 2). This would indicate that the divergence of peripheral and central nicotinic receptors happened very early during vertebrate evolution, as indicated also by the phylogenetic tree depicted in Fig. 14. Amino terminal amino acid sequencing analysis of the other isolated subunits from optic lobe AcChoR did not yield any signal above the high background consistently present, indicating that these subunits had blocked $NH_2$-termini.

```
BRAIN  XEFETKLYKELLKNYNPLEXPVAXD

RAY    SEHETRLVANLLENYNKVIRPVEHH

EEL    SEDETRLVKLNFSGYNKVVRPVNHF

CALF   SEHETRLVAKLFEDYNSVVRPVEDH

CHICK  XEHETRLVDDLFRDYSKVVRPVENH
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Figure 13. Comparison of the sequence obtained for the subunit of $M_r$ 48 K of brain AcChoR (top) and the amino terminal sequences of known α-subunits of peripheral AcChoR from Torpedo electroplax (25). Electrophorus electroplax and muscle (26, 20) and calf (1).

Table 2

| | chick | ray (25) | eel (26) | calf (1) | human (28) |
|---|---|---|---|---|---|
| α | 35 (27) | 48 | 43 | 39 | 39 |
| β | 38 (*) | 30 | 39 | 35 | - |
| γ | - | 26 | 30 | - | - |
| δ | 28 (*) | 32 | 24 | - | - |

% Identity between the sequence obtained for the lightest component of chick brain AcChoR and the amino terminal segment of subunits from other peripheral AcChoRs.

*Unpublished observations.

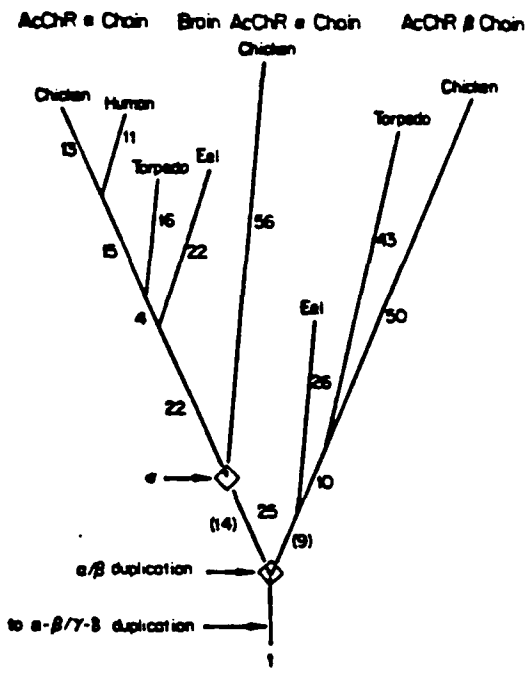


Figure 14. A phylogenetic tree generated from the amino terminal sequence data of the known α and β subunits of peripheral AcChoRs (1, 7, 20, 25, 26, 27) by using the best fit matrix method (36). Each branch point represents a nodal or ancestral sequence The numbers associated with each branch length represent the "accepted point mutations" (PAMs) per 100 amino acid residues that occurred in generating the contemporary AcChoR subunits. The position of the α/β duplication is arbitrarily located along a 25 PAM segment separating the ancestral sequences of modern α and β subunits. The * indicates the divergence of the α-subunit of the brain AcChoR from the ancestor of the peripheral α-subunits.

In the absence of sequencing data, other methods had to be used to identify the other peptides as AcChoR subunits. The peptide of apparent $M_r$ 54,000 has already been shown (46) to be the site of specific alkylation by the affinity reagent bromoacetylcholine (BrAcCho) and is therefore part of the receptor since it must contain at least part of one of the binding sites for cholinergic ligands. In the case of peripheral AcChoR and under similar conditions, only the α-subunit is labeled by BrAcCho (49). However, since multiple ligand binding sites have been shown to exist at least on Torpedo AcChR (52-54) and also since BrAcCho itself can label other Torpedo AcChoR subunits using different labelling conditions (unpublished observations), it is reasonable to conclude that in the case of the highly divergent CNS receptor a different subunit can be more easily labelled by BrAcCho. Likewise $^{125}$I-α-BTX can be cross-linked to the optic lobe 54K subunit by using dimethyl suberimidate (46 and unpublished observations). Hence, the 54K subunit carries a high-affinity site for ACh and for α-toxin, as is known for the α-subunit of the peripheral AChoR (2).

Further evidence that other polypeptides are components of the AChoR was obtained by virtue of their immuno-reactivity. For this test, we used a monoclonal antibody, 7B2, raised against chick muscle AChoR which has been shown to give distinct cross-reaction with the α-toxin complex of the AChoR purified from chick optic lobe (55). Purified optic lobe AChoR was immunoprecipitated by antibody 7B2, with complete

removal of toxin-binding activity from solution, using the methods of Mehraban et al. (55). Optic lobe AChoR was labelled with $^{125}I$, immunoprecipitated by 7B2 and the precipitate was extracted in SDS solution and analyzed. Three receptor bands were present which corresponded to the 54, 62 and 66K subunits observed upon SDS gel electrophoresis of non-iodinated receptor. The subunit of lowest $M_r$, for which sequence data have revealed its correspondence with the α-subunit of peripheral AcChR, was not evident upon SDS gel electrophoresis of the antibody precipitates of iodinated receptor; this was most likely due to the lability of this subunit as discussed above. Iodination also caused some breakdown of the two higher $M_r$ subunits which appeared weaker upon fluorography of $^{125}I$-labeled AcChR when compared with Coomassie or silver stained gels of non-iodinated AcChoR. The subunit of $M_r$ 72K was not precipitated and cannot therefore be identified as an AcChoR subunit.

The data reported here permit the identification of the 48K and 54K polypeptides as two different subunits of the central AcChoR. In the absence of sequencing data it is not possible to determine whether the other two protein bands present in $NaDodSO_4$ gels of purified CNS receptors having $M_r$ of 62 and 66K represent different subunits or different proteolytic products or of post-translational modifications of one subunit. The presence of blocked amino termini does not allow determination of the stoichiometry of these polypeptides in the receptor molecule.

These results establish that the CNS α-bungarotoxin binding protein is indeed a nicotinic binding protein similar to those found in muscle and electric organ. Brain and muscle AcChoR from the same species, although homologous, must be encoded by different genes and must have originated from the same ancestral gene. Since the similarity between α-subunits of central and peripheral receptors from the same animal is much less than between α-subunits of peripheral AcChoRs from different animals it may be concluded that the central and peripheral nicotinic receptors diverged very early during vertebrate evolution. These divergencies from an ancestral common structure explain well the pharmacological characteristics of these receptors which only partially overlap. Similarly the partial structural identity explains why in certain cases polyclonal and monoclonal antibodies raised against peripheral AcChoRs failed to recognize central receptors (55, 56) and antibodies against chick optic lobe receptor did not bind to peripheral AcChoR from chick or from Torpedo californica (54). Our data do not exclude the possibility that within the nervous system different nicotinic receptors may exist. This possibility is supported by the following findings: (a) neuronal proteins exist which do not bind α-BTX but bind anti-muscle AcChoR antibodies as well as other α-BTX-like snake venom toxins (57-59) and (b) in chick sympathetic ganglia two nicotinic receptors with slightly different pharmacological specifities exist both of which bind α-BTX-like toxins but only one of them is functionally blocked as a consequence of α-BTX binding (60, 61).

It has been reported that antibodies raised against chick optic lobe receptor cross-react not with chick muscle AcChoR but with the receptor from the PC12 neuronal cell line (62). This raises the possibility that neuronal nicotinic receptors are highly conserved proteins and that the genes encoding chick brain AcChoR, whose isolation will be greatly facilitated by the reported sequence data, will permit the isolation of mammalian brain AcChoR genes.

## Experimental

### 1. Sealed Vesicle Preparation

Torpedo californica electric tissue is treated as previously described (20) and the vesicles enriched in AcChR identified by $^{125}I$ α-Butx binding (63). Further purification

is achieved by base treatment (20, 64) and intact vesicles separated from membrane pieces and leaky vesicles by rapid centrifugation in 8-20% sucrose gradients (45).

2. Synthesis of Labeled Amino Acids

(a) [$^3$H] methionine amide. Homocysteine thiolactone (1 mmole) is dissolved in liquid ammonia and allowed to sit for one hour to allow the ammonia to react and form homocysteine amide. Sodium metal is added until a blue color persists for several minutes to ensure that the product is in the reduced (-SH) form, [$^3$H] methyliodide (1 mmole) of specific activity 100 mCi, $mM^{-1}$ is then added and allowed to stir for one hour, excess unlabelled methyl iodide (ten fold) is added and allowed to react for one hour with reflux using a cold finger. The ammonia was allowed to evaporate overnight, yielding a white solid. This was dissolved and the pH adjusted to 9.5 and extracted with dichloromethane using a continuous extraction apparatus, for two days, until essentially quantitative extraction of $^3$H had occurred. The solvent was removed and the [$^3$H] methionine amide recovered as an off-white solid. It was dissolved in water and titrated to pH 5 with diluted HCl and hyophilized. Amino acid analysis revealed that the $^3$H incorporated was associated with methionine amide. Specific activities of different preparations ranged from 22-52 mCi, $mM^{-1}$.

(b) [$^3$H] methionine. This was synthesized from homocysteine in sodium/liquid ammonia and [$^3$H] methyl iodide as described above and isolated by crystallization as described long ago (65).

3. Carboxypeptidase Y Labeling of Proteins, including AcChR

The normal degradative reaction of proteins occurs at around pH 7. It is possible to reverse the reaction at high pH, using high enzyme concentration and a high nucleophile concentration, i.e., [$^3$H] methionine or methionine amide. Application of this method to small peptides has been described (32). Labeling of proteins is generally done using 0.5 M carbonate-bicarbonate buffer, pH 9.5, 0.1-0.2 M nucleophile and 1 µg/ml carboxypeptidase Y.

4. Carboxypeptidase Y Catalysed Degradation of AcChR Enriched Vesicles at Neutral pH 7. Receptor enriched vesicles (125 µg receptor protein) was treated with cp-y (12.5 µg) in 100 µl of 100 mM sodium phosphate buffer, containing 1 mM EDTA at pH 7.0 for varying periods of time. Analysis of the conversion of receptor dimer to receptor monomer was conducted by running the mixtures in sucrose gradients (34).

5. Synthesis and Purification of [7(n)-$^3$H] Cholesterol Diazoacetate

Cholesterol diazoacetate was synthesized and purified by the method of Keilbough and Thornton (28). A similar method was used to synthesize [7(n)-$^3$H] cholesterol diazoacetate using [7(n)-$^3$H] cholesterol (12 Ci/nmol) purchased from ICN Pharmaceuticals, Inc. 5 µmol cholesterol was added to 5 mCi of [7(n)-$^3$H] cholesterol in benzene (1 mCi/ml). The solvent was removed under reduced pressure at 22°C. The cholesterol was dissolved in 14 µl of $CH_2Cl_2$:THF (1:1) containing 5 µmol of redistilled trietylanine on ice. 5.2 nmol of glyoxylic acid choloride p-toluene sulfonyl hydrazone was added in 17 µl of $CH_2Cl_2$ cooled to 0°C. After 30 minutes in the dark on ice, 5 µmol of TEA in 5 µl of $CH_2Cl_2$ was added. The solution was brought to 22°C. After 30 minutes, the solvent was removed under reduced pressure at 22°C. The residue was extracted twice with 100 µl of toluene. The extracts were applied to a .5 x 4 cm silica gel 60 column (Merck) and eluted with toluene. Toluene was removed under reduced pressure and the diazoacetate was taken up in ethanol for determination of the specific activity and introduction into membranes.

6. Effects of Cholesterol and Phospholipids on AcChR Function

Lipids were extracted from crude Torpedo membranes as described (66). Phospholipid phosphate was assayed by the method of Ames (1966) and the level of cholesterol was determined enzymatically using the Sigma diagnostic Kit (no. 350). Four lipid fractions were prepared. The lipid fraction extracted from crude membranes (hereafter referred to as "whole lipid") had the same phospholipid composition but half the cholesterol level found in AcChR-rich membranes. A second fraction was supplemented with cholesterol to bring it up to the native level and is referred to as "whole + chol. lipid." A whole lipid fraction was extracted with acetone (67) to remove the cholesterol. Analysis showed that this fraction ("X-lipid") was 94% depleted of cholesterol. Finally, equal amounts of "whole" and "extracted lipid" were combined to produce a fraction with 1/4 the native level of cholesterol ("whole + X-lipid").

All lipid fractions were dissolved in chloroform, dried to a film along the wall of a tube with a stream of argon, then dried under vacuum overnight. Buffer was added to give a concentration of ∿35 mg P-lipid/ml. The mixtures were sonicated for 30 minutes under the same conditions described above to produce a dispersion of small unilamellar vesicles.

Synthetic phospholipid vesicles were made in the appropriate proportions to a concentration of 40 mg/ml plus 20% cholesterol (8 mg/ml). The mixtures were sonicated for about 10 minutes as described.

The following procedure was used to fuse AcChR-rich membranes with phospholipid vesicles. AcChR membranes and an excess of vesicles were mixed and solid PEG added to 30% (w:w). This was incubated tor 30 minutes at room temperature with intermittent stirring to dissolve the PEG. The mixture was then slowly diluted ∿20X (over the course of 1 minute) with buffer lacking sucrose. The vesicles were pelleted by centrifugation at 35,000 RPM for 15 minutes in a Beckman 70.1 Ti rotor. The pellet was resuspended in buffer then subjected to two cycles of freezing and thawing. Finally, the vesicles were sonicated as described above for 20 seconds.

Membrane fusion was analyzed by equilibrium density centrifugation in 10-40% sucrose gradients. Gradients were prepared in Beckman quick-seal tubes (5.1 ml) and after loading ∿200 µl samples, centrifuged in a VTi 80 rotor for 2.5 hours at 50,000 RPM. The tubes were eluted by puncturing the bottom and fractions counted for [$^{14}$C]-PC or [$^{125}$I]-α BuTx.

The kinetics of carbamylcholine-induced cation transport were determined by the stopped-flow method (19) in which the fluorescence of the probe, PyTSA (68) loaded within the vesicles is quenched due to the influx of $Tl^+$. The excitation wavelength was 370 nm and an emission filter (Corning 3-75) was used. Fluorescence decay traces were analyzed by using a two exponential equation. Titration data of carb-induced flux was fit to a binding curve assuming two ligand binding sites.

AcChR membranes were labeled with IANBD as described except that incubation with the reagent was allowed to proceed overnight at 4°C. After removal of excess reagent by gel filtration the membranes were concentrated by centrifugation for 15 minutes at 35,000 RPM in a 70.1 Ti rotor. These were then fused with lipid vesicles as described earlier. The carb-induced fluorescence increase was recorded on a Perkin-Elmer MPT-4 spectrofluorimeter using excitation and emission wavelengths of 482 and 537 nm, respectively. Titration data were fit to a simple binding curve assuming one binding site. Electron microscopy was performed on a Philips EM 201. Samples were negatively stained with 1% uranyl acetate.

7. $^{51}Cr$ Labeling of Acetylcholine Receptor

$^{51}Cr^{+++}$ was converted to $^{51}Cr^{++}$ by treatment with zine-amalgam as described in (75). Receptor enriched vesicles (150 µg protein in 0.5 ml cacodylate buffer, pH 6.1) were added to 0.1 ml of the $^{51}Cr^{++}$ solution so that the final Cr concentration was 1 mM. For oxidation to $Cr^{+++}$ 0.5 ml of the same buffer saturated with $O_2$ was added with or without acetylcholine.

8. Structural Studies of Acetylcholine Receptors

(a) Preparation of AChoR. Chick optic lobes or brains (i.e., brain minus optic lobe) were homogenized (glass/teflon, 10 passes) in 10 vol buffer 2 [50 mM Na phosphate (pH 7.0), 5 mM EDTA, 1 mM EGTA] containing also 1 mM PMSF, 100 µg/ml bacitracin, 25 µg/ml soybean trypsin inhibitor, 1 mM benzamidine and (except where noted) 0.5 mM N-ethyl maleimide (NEM) or 2 mM iodoacetamide. The pellet collected at 100,000 g was homogenized in 4 vol buffer 2 containing the protease inhibitors less NEM. Triton X-100 was added to a final concentration of 1% and the extract was shaken at 0°C for 1 hr. The supernatant (100,000 g, 45 min) was filtered through glass wool, roller-skated at 4°C for 3 hr with 2 ml Sepharose-α-BTX (concentration of coupled toxin ∿0.5 mg/ml resin) per 100 ml of supernatant. The beads were washed rapidly in a column, first with 30 vol of buffer 2 containing 0.2% Triton X-100 and protease inhibitors, then 100 vol of the latter medium containing 1 M NaCl, and finally with 60 vol of buffer 2 containing 0.2% Triton. The beads were extracted (a) with 1.5 vol of 1 M carbachol in buffer 2 containing 0.2% Triton with rotation for 12 hr at 4°C, and combining the extract with a second 2 hr extract or (b) with 1.5 vol 62 mM Tris/HCl (pH 6.8), 3% SDS and 5% mercapto-ethanol, rotating 1 hr at room temperature. Extract (a) was dialyzed against several changes of 10 mM Na phosphate, 50 mM NaCl, 0.1% Triton (pH 7.4). $NadodSO_4$ and mercaptoethanol were added to final concentrations of 1.5% and 2.5% respectively and the samples were dialyzed extensively against 16.7 mM Tris HCl (pH 6.8), 0.05% $NaDodSO_4$ (pH 6.8). Extract (b) was dialyzed similarly.

(b) Purification of AcChoR subunits. The peptides present in preparations of purified AcChoR (50-200 pmol of toxin binding sites) were isolated by $NaDodSO_4$ gel electrophoresis, recovered by electroelution and electrodesalted as described (70).

The purity and integrity of the isolated subunits were assessed by gel electrophoresis (71). The protein bands were visualized by silver staining (72). The purified subunits were lyophylized and stored frozen.

(c) Amino-terminal amino acid sequence analysis. The lyophilized samples were dissolved in glass-distilled water and 30 µl were loaded on a polybrene coated glass filter disk in a gas phase sequencer (Applied Biosystems) and submitted to amino-terminal sequence (ATAS) analysis by automated Edman degradation (73). The polybrene coated disk had been precycled using 10 cycles of automated Edman degradation. For each analysis 5-20 pmole of protein were used. PTH amino acids were identified by HPLC by using an IBM Cyano column (74). Typical repetitive yields for these ATAS analyses were between 93 and 96%.

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