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

ANNUAL REPORT

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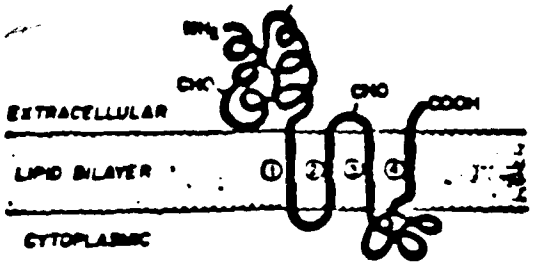
Background

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 $\alpha_2 \beta \gamma \delta$ 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 6×10^6 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.

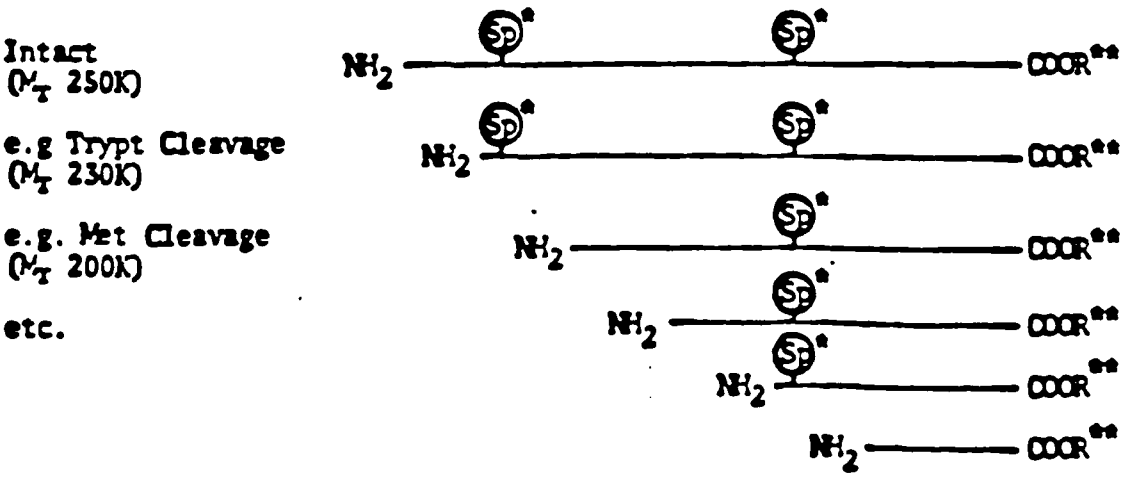
Approach to the Problem

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

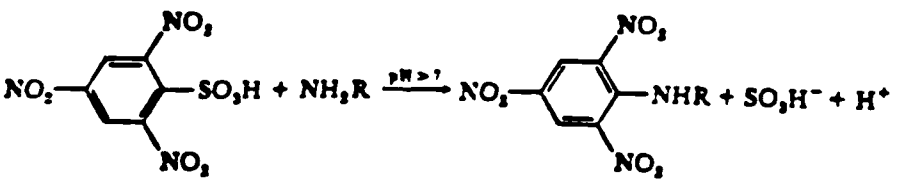
label. (Another approach is to label the protein separately, do parallel autoradiographs and compare visually—and confirm identity by microsequence analysis if necessary.

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 -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 electrophoresis. 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. Chemical labeling of the receptor from the bilayer. Previously we have reported in preliminary form 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.

2. Exposure of acetylcholine receptor to the lipid bilayer

The nicotinic AChR isolated from the electric organ of T. californica is a pentameric complex of four homologous subunits with apparent molecular weights of 40K, 50K, 60K, and 65K in a stoichiometry of 2:1:1:1(32). As AChR is an ion channel, studies directed at the transmembrane nature of the subunits are crucial to our understanding of communication through the nicotinic synapse. A unique opportunity to investigate the transmembrane nature of an ion channel is afforded by the availability of membrane fragments greatly enriched in AChR prepared from Torpedo electric organ (33).

The exposure of T. californica AChR on both extracellular and cytoplasmic sides of the post-synaptic membrane has previously been shown using tryptic hydrolysis(34,35). Trypsin added to sealed vesicles, 95% of which had the synaptic surface facing the external medium, degraded all four subunits. A freeze-thaw procedure was used to load trypsin within vesicles, also resulting in proteolysis of all four subunits. The more recent

experiments have shown both cytoplasmic and extracellular treatment with trypsin, although causing significant subunit degradation, does not alter agonist induced cation flux properties of the receptor (35). This reinforces the conclusion that proteolysis occurs at tryptic cleavage sites exposed on both sides of the membrane in each of the subunits rather than an artifactual exposure of tryptic cleavage sites caused by degradation and perturbation of the AChR-lipid complex.

Although it is evident that all of the subunits span the post-synaptic membrane, whether all four subunits are exposed to the lipid bilayer has not been established. In this communication, we have investigated the exposure of AChR to the hydrocarbon core of the lipid bilayer using the photolabile probe [^{3}H]adamantanediazirine. This hydrophobic probe has previously been shown to efficiently label both unsaturated and saturated membrane lipids (36) and has subsequently been used to selectively label regions of integral membrane proteins exposed to the lipid bilayer (37-40).

The distribution of [^{3}H]adamantanediazirine between the aqueous phase and AChR enriched membranes was determined by incubation of the hydrophobic probe with membrane fragments which were then pelleted by centrifugation. The partition coefficient for the diazirine between the aqueous buffer and the membrane fragments defined as (mol ligand bound/mg prote :)/(mol free ligand/µl external solution) is 1320. Assuming a density for the hydrocarbon core of 0.75 g/ml and using published values for the protein-lipid ratio in AChR enriched membrane fragments (41,42), the ratio of diazirine in the hydrocarbon core to that in the surrounding aqueous phase is ∿2000. This result indicates [^{3}H]adamantanediazirine partitions into the lipid bilayer where it can interact with regions of membrane proteins in contact with the lipids.

Irradiation of AChR enriched membrane fragments equilibrated with [^{3}H]adamantanediazirine resulted in labeling of all four subunits (fig. 2). In addition, there was a peak of radioactivity corresponding to a polypeptide of Mr 30K, which was most likely a proteolytic fragment. There also was incorporation of radiolabel into a 90K dalton protein when it was present in the membrane preparations (data not shown). The large peak of radioactivity which comigrates with the dye front corresponds to radiolabeled lipids. In all of the experiments, between 30 and 40% of the radiolabel was incorporated covalently into membrane fragments upon photolysis. Of that, about 50% was incorporated into protein components with the remainder covalently attached to membrane lipids.

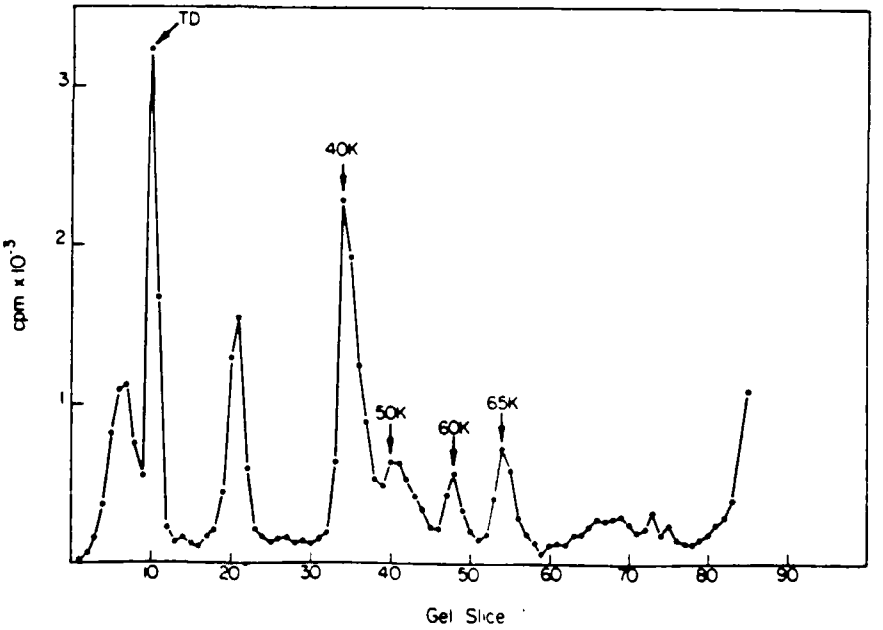


Fig. 2: Polyacrylamide gel electrophoresis scan of AChR enriched membrane fragments (65 ug protein) labeled with [^{3}H]adamantanediazirine (TD = tracking dye).

The apparent molar ratio of radiolabel incorporation into the 40K, 50K, 60K and 65K subunits was about 3:1:1:1, i.e. a stoichiometry of 6:1:1:1. This ratio was determined by purifying the subunits using preparative electrophoresis, after which the specific incorporation of radiolabel into each of the subunits was determined by amino acid analysis and scintillation

counting. The greater reactivity of the 40K subunit could be due to either its amino acid composition or a greater surface exposure. Dynamic interactions with other receptors or membrane proteins could also affect the time-averaged surface exposure of a particular subunit to the lipid bilayer.

The effect of carbamoylcholine, which causes a conformational change which has been correlated with physiological desensitization (43-45), on the photoincorporation of radiolabel into the subunits was investigated. Inclusion of 10 μM carbamoylcholine in the buffer did not alter the incorporation of radiolabel into the subunits. Likewise, saturation of AChR with α-BgTx did not affect the radiolabel distribution among the subunits. Photolabeling of both the 9S monomer and the 13S dimer in membrane fragments also resulted in indistinguishable distributions of radiolabel among the subunits.

The inclusion of 20 mM reduced glutathione in the buffer did not alter the ratio of photoincorporation of radiolabel among the subunits, although the total incorporation of radiolabel into the subunits decreased by about 10%. As glutathione is an aqueous soluble scavenger of carbenes, this experiment confirms that the labeling occurs in regions of AChR exposed to the lipid bilayer.

The possibility that photolabeling by [^{3}H]adamantanediazirine was occuring at high affinity saturable binding sites was investigated. The membrane fragments were labeled with 100 μM adamantanediazirine and then labeled with 18 μM [^{3}H]adamantanediazirine. This prelabeling did not alter the total covalent incorporation into membrane fragments or the incorporation of radiolabel into the AChR subunits, indicating that the diazirine interacts non-specifically with AChR after partitioning into the lipid bilayer.

The exposure of AChR subunits to the lipid bilayer has previously been investigated using hydrophobic photolabile probes, yielding conflicting results. Irradiation of 5-[^{125}I]iodonapthyl-1-azide equilibrated with AChR enriched membrane fragments isolated from T. californica exclusively labeled the 40K subunit (46). A similar reagent, [^{3}H]pyrenesulfonyl azide labeled polypeptides of 48K and 55K daltons in membranes (47,48). This variability in labeling may have arisen from preferential reactivity of different nitrenes with particular subunits.

[^{3}H]Adamantanediazirine, which generates a carbene upon irradiation, was proposed as an alternative to aryl nitrene probes by Bayley and Knowles (36). After finding that [^{3}H]adamantanediazirine labeled both unsaturated and saturated membrane lipids more efficiently than aryl nitrenes (36,49), the probe was subsequently used to selectively label regions of integral membrane proteins in contact with the hydrocarbon core of the lipid bilayer (37-40). Our results indicate that [^{3}H]admantylidene may be a more effective hydrophobic probe than aryl nitrene probes.

AChR is a pentamer of homologous subunits (32), all of which span the membrane (34,35). Recently elucidated sequences of cDNAs for these subunits indicate that there are four hydrophobic regions conserved in each subunit (50-55). In this communication, we have demonstrated experimentally that all four subunits are exposed to the hydrocarbon core of the lipid bilayer. It will be of interest to determine whether the labeling occurs at the sites predicted from the sequence analysis or at other sites as well.

3. Direct Binding of an Agonist to Low Affinity Sites

From all the above considerations, muscle and neuronal AcChoRs appear to be complex proteins carrying multiple homologous domains and multiple binding sites for cholinergic ligands, possibly one for each subunit. All these sites could bind agonists and antagonists, but with a spectrum of affinities and functions different in different AcChoRs. At low ligand concentration only high affinity sites were revealed, both in terms of their number and of their effect(s). Only using special ligands with broad specificity like α-Dtx or suitably high concentration of the other ligands can one reveal and study the low affinity sites which are associated with channel activation and therefore normal physiological function. At equilibrium, when the receptor is desensitized AcCho binds with high affinity (Kd~10 nM) to two sites per AcChoR (see 2). Affinity labelling techniques using the alkylating agonist, bromoacetylcholine (56-59) have shown that these high affinity sites are associated with each of the two α-subunits. Economy of hypotheses led to the assumption that these two sites are the only sites for agonists and that their occupancy leads in a sequential fashion to both channel opening and to desensitization. However, despite extensive studies of the kinetics of ligand binding to these sites all observed transitions have been too slow to be involved in channel opening and the affinity of these sites, even in the resting state of the receptor, is much higher than apparent affinities measured in flux experiments (see 2).

A perhaps simpler explanation for the discrepancy between dissociation constants measured in direct binding and ion flux experiments is that the AcChR has multiple binding sites and that channel opening occurs as a result of agonist interaction with sites that are not readily measured *in vitro* since they have intrinsically low affinity in agreement with the dose dependency of ion flux. Such a model was originally suggested from the demonstrated sequence homology of the subunits (3-9) which suggests that homologous binding domains may exist on some or all of the subunits. Recently we have obtained considerable experimental evidence in strong support of this model.

Conclusive evidence for the existence of multiple binding sites comes from the demonstration described above that whereas α-bungarotoxin (α-BuTx) and the long α-neurotoxin from *Naja naja siamensis* venom bind pseudo-irreversibly to two sites on the AcChoR, which are presumably those associated mainly with the α-subunits (60,61), the long α-neurotoxin from the venom *Dendroaspis viridis* binds with high affinity to four sites (see above). Agonists also bind to the *Torpedo* AcChoR with multiple affinities. Using the fluorescence of a probe, IANBD, which was covalently bound to the receptor protein without measurable perturbation of receptor function we monitored agonist binding to low affinity sites which are distinct from the high affinity sites on the α-subunits which are labelled by bromoacetylcholine (59,62-64). The binding constants for these low affinity sites were in excellent agreement with the midpoints of dose-response curves for ion flux and this together with other characteristics of agonist binding to these sites including the rapidity of the associated conformational change (k_{max}~600 s^{-1} for AcCho) strongly suggest their involvement in channel opening.

In the past the only evidence for the existence of these low affinity sites, although substantial, was indirect since it was dependent on the fluorescence enhancement of a covalently bound fluorophore. It was not possible to directly measure agonist binding to these sites because although with *Torpedo* AcChoR one may obtain receptor concentrations in excess of 10 μM the affinities are too low (even

in the best case, that of AcCho, the Kd was ~100 μM) to allow direct binding measurements with radiolabelled ligands.

One agonist which in electrophysiological studies has been found to be effective at lower concentrations than AcCho is suberyldicholine (SdChoR, 65,66). This is a bifunctional ligand having two quaternary ammonium groups separated by a 14-atom chain. Using a rapid kinetic method to monitor thallium influx (19) in AcChoR-enriched membrane vesicles we have found that the midpoint of the flux response occurs at 1-3 μM (unpublished observations). For this agonist, as for all other previously examined, there is serious quantitative discrepancy between this affinity estimated in flux experiments and its equilibrium dissociation constant for binding to its high affinity sites which has been estimated to be 10 nM from measurements of inhibition of the initial rate of ^{3}H-α-neurotoxin binding (66). From these results it may be predicted that SdCho binds much more tightly to the low affinity site(s) involved in channel activation than other agonists facilitating direct measurements of "low affinity binding" using radiolabelled SdCho.

Binding of SdCho to NBD-Labelled AcChoR

The binding of SdCho to NBD-labelled membrane preparations was accompanied by an enhancement of fluorescence which was saturable in the micromolar range. Dissociation constants obtained from this fluorescence enhancement lay in the range 1-3 μM in excellent agreement with the midpoint of the flux response. This is consistent with results we have obtained for all other agonists examined (63,64) where the fluorescence of covalently bound NBD reflects binding to low affinity sites which exist at equilibrium and are likely to be important in channel activation. The fluorescence changes induced by SdCho were, however, more difficult to measure than when other agonists such as AcCho or CarbCho were used because of a smaller quantum yield change. The reasons for this are discussed below.

Binding ^{3}H-ScCho to AcChoR

Figure 15 shows a typical Scatchard plot of ^{3}H-SdCho binding to the membrane-bound AcChoR as measured in equilibrium dialysis experiments. At all concentrations of AcChoR used (0.1-5.0 μM) binding of ^{3}H-SdCho was saturable at a number of sites equal to the number of sites for ^{125}I-α-BuTx, i.e., two sites per receptor molecule. In each case Scatchard plots of the data were linear suggesting that binding was to a homogeneous population of sites with a dissociation constant of 14±5 nM (mean of 12 experiments). These results were surprising because no heterogeneity of binding was apparent and no confirmation of the existence of the low affinity site(s) measured in the equilibrium fluorescence titrations was obtained. However, competition experiments with other ligands have shown that the binding of ^{3}H-SdCho is more complex than the linear Scatchard plots suggest.

^{3}H-SdCho Binding in the Presence of High Concentrations of CarbCho

When ^{3}H-SdCho binding to AcChoR-enriched membrane fragments (1 μM in α-BuTx sites) was measured in the presence of a concentration of unlabelled CarbCho sufficient to saturate the high affinity sites the results revealed that contrary to the simple appearance of the Scatchard plot (Fig. 3) the binding of SdCho is complex and involves binding sites of different affinities. In the absence of CarbCho the binding of ^{3}H-SdCho was virtually stoichiometric (Fig. 4) as would be expected from its estimated high affinity (Kd = 14 nM) and was again saturable at two sites per

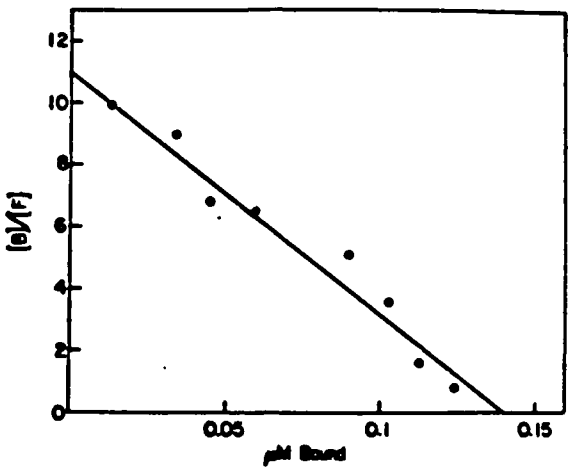


Figure 3. Scatchard plot of ^{3}H-SdCh binding to membrane-bound AcChoR (0.15 μM in α-BuTx binding sites). Solid line is least squares fit of the data giving Kd = 12.7 nM and concentration of ^{3}H-SdCho sites-0.14 μM.

AcChoR. However, when the receptor was incubated with 100 μM Carb, a concentration well in excess of its Kd for the two high affinity sites (~100 nM) instead of being completely inhibited from binding ^{3}H-SdCho could still bind but with reduced affinity. Fig. 4a shows that in the presence of 100 μM CarbCho the measured amount of ^{3}H-SdCho bound was much greater than would be expected if ^{3}H-SdCho with a Kd of 14 nM was merely displacing CarbCho from its high affinity sites. The expected displacement is indicated in the Figure and if this is subtracted from the observed amount of ^{3}H-SdCho bound, the binding data may be represented as a linear Scatchard plot (Fig. 4b) in which there is one ^{3}H-SdCho site per AcChoR having a Kd of 0.84 μM. Thus apparent saturation of the high affinity sites by another ligand seems to have "unmasked" a low affinity binding site for ^{3}H-SdCho and the affinity of this site is in good agreement with estimates from ion flux or fluorescence titration experiments.

Direct binding measurements therefore suggested that the AcChoR has only two nigh affinity sites for ^{3}H-SdCho but a lower affinity site was revealed when the former were presumably saturated by CarbCho. The question remains as to why the low affinity binding of ^{3}H-SdCho is not observed in the absence of other ligands. Fluorescence titrations of NBD-labelled AcChoR have shown that ^{3}H-SdCho occupies this site at equilibrium and therefore this binding must occur but somehow be obscured by other events to give rise to a linear Scatchard plot.

Equilibrium Binding of ^{3}H-SdCho and/or ^{14}C-AcCho

The properties of ^{3}H-SdCho binding to the AcChoR were clarified when its binding was measured in the presence of ^{14}C-AcCho. The binding of ^{14}C-AcCho to membrane-bound AcChoR has been measured by equilibrium dialysis and in

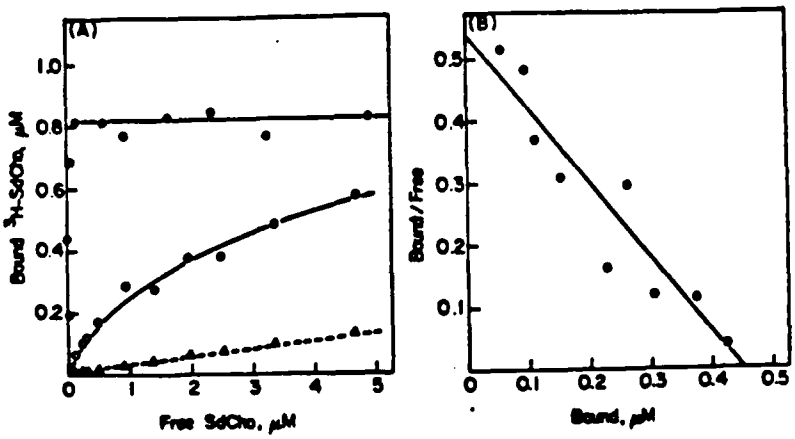


Figure 4. Effect of CarbCho on ^{3}H-SdCho binding to membrane-bound AcChoR (1 μM in α-BuTx binding sites). In (A) SdCho binding was measured in the absence (●–●) or presence (O–O) of 100 μM CarbCho., The lowest curve (Δ–Δ) is a theoretical one based on the expected amount of ^{3}H-SdCho bound if it competed only for the high affinity binding sites and is calculated using Kds for SdCho and CarbCho of 14 nM and 100 nM, respectively, and a total concentration of binding sites of 0.81 μM. (B) Scatchard plot of the data in (A) for SdCho binding in the presence for 100 μM CarbCho after subtraction of the amount of ^{3}H-SdCho bound due to direct displacement of CarbCho from its high affinity binding sites. Solid line is linear least squares fit to the data giving Kd = 0.84 μM and concentration of ^{3}H-SdCho sites = 0.45 μM.

appearance is very similar to that of ^{3}H-SdCho. Scatchard plots were linear suggesting the presence of two high affinity ^{14}C-AcCho sites per AcChoR having a Kd of 10 nM. In order to investigate whether the "unmasked" low affinity binding was due to some perturbation of the high affinity sites we have measured the binding of ^{3}H-SdCho in the presence of a constant concentration of ^{14}C-AcCho to allow parallel measurement of the behavior of both ligands. Figure 5 shows the binding of ^{3}H-SdCho to AcChoR (2.5 μM in α-BuTx sites) in the presence of 5 μM ^{14}C-AcCho. As the concentration of ^{3}H-SdCho was increased again the amount bound was greater than expected if there was merely direct competition between the two ligands for the two high affinity binding sites. This unmasked low affinity binding of ^{3}H-SdCho was, however, paralleled by a loss of bound ^{14}C-AcCho so that the total number of ligand binding sites occupied remained constant at two per AcChoR molecule. It appears therefore that occupancy of a low affinity site by ^{3}H-SdCho displaces ^{14}C-AcCho from one of its high affinity sites.

Effect of Covalently-Bound Bromoacetylcholine on ^{3}H-SdCho Binding

We have previously reported that covalent modification of the two high affinity sites on the α-subunits by alkylation of the reduced receptor by BrAcCho did not affect the low affinity binding of AcCho as measured by fluorescence changes of NBD-labelled preparations (62,64) suggesting that for this agonist there is no interaction between high and low affinity sites. In contrast prelabelling of the receptor by BrAcCho

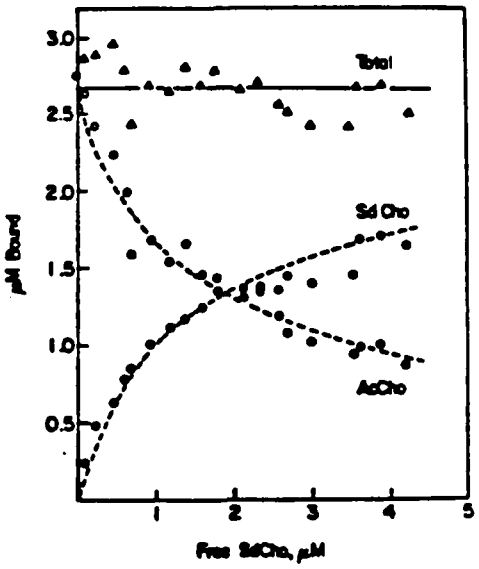


Figure 5. Effect of increasing ^{3}H-SdCho concentration on binding to membrane-bound AcChoR (2.5 μM in α-BuTx binding sites) in presence of constant concentration of ^{14}C-AcCho (concentration of free ^{14}C-AcCho~4 μM). The amount of bound ^{3}H-SdCho and ^{14}C-AcCho was measured as described in the text. The dotted lines are calculated fits using the experimental data and the model described in the text in which there are originally two high affinity sites occupied by ^{14}C-AcCho (Kd = 10.4 nM) but occupancy of one low affinity site by ^{3}H-SdCho (Kd~1 μM) eliminates one high affinity site and there is also direct displacement of ^{14}C-AcCho from its high affinity sites by high affinity binding of ^{3}H-SdCho (Kd = 13.8 nM).

completely inhibited the binding of ^{3}H-SdCho to both its high and low affinity sites. This, together with the apparent loss of one of the high affinity sites with increasing SdCho concentration raises the possibility that the low affinity binding of SdCho may be stabilized by deriving some binding energy by cross-linking one of the "primary" sites on the α-subunits. SdCho is a relatively large molecule with its bis-quaternary ammonium groups separated by a distance of ~23 Å. Both positive charges are likely to be involved in binding to negative subsites in the receptor and it it is possible that these subsites may be on different subunits.

If the low affinity binding of SdCho is stabilized by such crosslinking this may explain why this agonist is effective at low concentrations. It is likely that the conformational change which leads to channel opening occurs as a result of its interaction with the "secondary" binding site since SdCho binding causes an enhancement of fluorescence of NBD-labelled AcChoR which seems to be relevant to channel opening events and NBD does not reflect binding to the well documented sites on the α-subunits (unpublished observations). However, by having multiple

interactions with the protein, the affinity for SdCho will be increased thus shifting the midpoint of the flux response to lower concentrations.

Stoichiometry of Low Affinity Binding Sites

It was somewhat surprising that only one low affinity binding site for ^{3}H-SdCho was detected because detailed studies of the AcCho-mediated flux response have shown that the flux rate has a sigmoidal dependence on concentration and a Hill coefficient of 1.7 (67). This indicates that the channel is more likely to be opened if two AcCho molecules are bound, in agreement with electrophysiological results (68) and leading to the prediction that there are two low affinity binding sites rather than one.

Evidence that SdCho is different from other agonists in its binding and functional responses has been obtained from a study of fluorescence changes of NBD-labelled AcChoR. Equilibrium fluorescence titrations using SdCho have been hampered by the low amplitude of the enhancement, which consistently was only ~50% of that induced by AcCho or CarbCho. The fluorescence data shown in Figure 4a provide an explanation for this low amplitude and show that while SdCho has only one low affinity binding site of Kd ~ 1 μM has two low affinity sites of equal affinity. When NBD-labelled AcChoR was titrated by SdCho until well beyond saturation of its binding site ([SdCho] = 100 μM) and the titration was continued with the other agonist a second site was present for CarbCho having a Kd identical to that measured in the absence of SdCho but with half of the amplitude change. Similar results were obtained with AcCho as the second ligand.

A more detailed examination of the effect of SdCho on NBD-labelled AcChoR has revealed that for this agonist also there is a second site but of much lower affinity having a Kd of ~2.5 mM (Figure 6b). Thus agonists such as AcCho and CarbCho appear to have two low affinity sites which accounts for the sigmoidicity of the flux response. On the contrary the two sites revealed by NBD fluorescence, bind SdCho with different affinities, the complexes having Kd's of 1 μM and 2.5 mM. Since SdCho mediates ion flux at micromolar concentrations it seems that the lowest affinity site does not need to be occupied for channel opening to occur. In this respect it is interesting that the Hill coefficient for SdCho mediated ion flux does not deviate from 1.0 and the maximum flux rate of ~50 sec^{-1} for this agonist is only ~10% of that for AcCho (unpublished observations).

Use of Two Fluorescent Probes to Obtain Structural and Conformational Information

As discussed above we have described the existence of additional low affinity binding sites for agonists on the *Torpedo* AcChR (69-70,60). These sites were initially revealed by fluorescence changes of a probe, IANBD, which was covalently bound to the receptor protein. The characteristics of binding to these hitherto unidentified sites, particularly the excellent agreement between dissociation constants obtained from fluorescence titrations of NBD-labelled receptor and from ion flux and the rapidity of the associated conformational change, led us to propose that occupancy of these sites is important in channel opening. The slower conformational changes associated with agonist binding to the well characterized α-subunit sites may occur in other parts of the molecule leading to channel closing and desensitization.

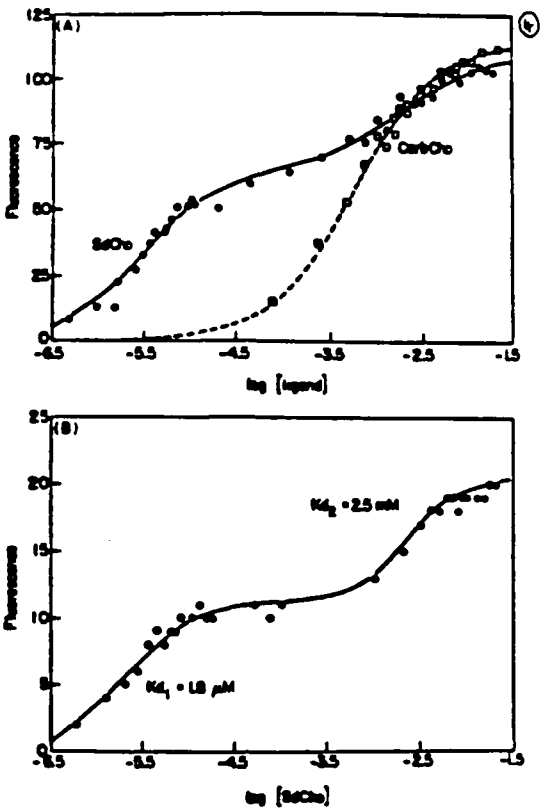


Figure 6. (A) Titration of NBD-labelled AcChoR by SdCho and CarbCho. Fluorescence enhancement occurring on addition of increasing concentrations of SdCho (●–●) gave a Kd of 1.9 μM. After saturation of this site with 100 μM SdCho the titration was continued with CarbCho (O–O) revealing an additional binding site for this ligand having a Kd of 1.6 M. In the absence of SdCho (–) CarbCho appeared to two sites having identical affinity (Kd = 0.7 mM). (B) Effect of SdCho concentration on fluorescence of NBD-labelled AcChoR. Solid line is best fit to two independent sites having Kds of 1.8 μM and 2.5 mM.

In this part of the report we describe the double labelling of the AcChR by two fluorescent probes one of which (IAS) we have previously used in studies of agonist binding (71,72) and which monitors binding to the high affinity sites on the α-subunits and one (IANBD) reflects low affinity binding as referenced above. We show that both classes of sites exist in the one preparation and that conformational changes associated with agonist binding to each site occurs in parallel and with different rates.

Localization of the IAS Label

Following mild reduction of the AcChR, alkylation by IAS results in a highly fluorescent receptor preparation whose fluorescence is enhanced in a specific and

saturable manner on the binding of agonists (71; Figure 7) or antagonists. Affinity labelling techniques have shown that each α-subunit carries a high affinity binding site for agonists and antagonists and close to these sites there is a reactive disulphide bond which may readily be reduced and labelled by reaction with affinity agents such as the agonist, bromoacetylcholine. Prelabelling of the AcChR by IAS partially inhibited the incorporation of ^{3}H-BrAcCh (71) showing that the fluorescent probe also reacts with this reduced disulphide near the high affinity binding sites. The results in Figure 7 show that the enhancement occurring on binding of ligands is due

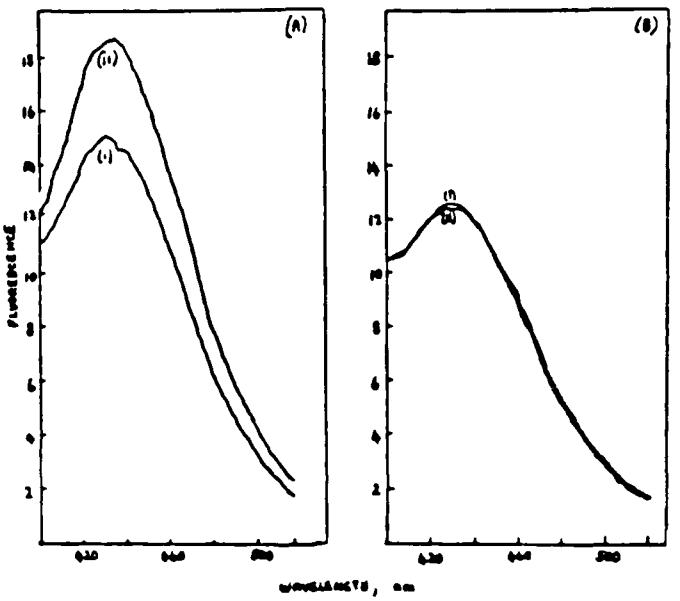


Figure 7.

specifically to the probe bound in this position. When the reactive disulphide near these high affinity sites is specifically protected from reduction by the presence of low concentrations of carbamylcholine although there is significant non-specific incorporation of IAS the fluorescence enhancement occurring on ligand binding is completely abolished. If Carb is included at the labelling step but not during the reduction there is a reduced but significant enhancement of fluorescence. The incorporation of IAS in this position does not perturb the equilibrium binding of agonists to their adjacent sites (see below) nor does it affect the ability of agonists to mediate ion flux (71). Prelabelling of the AcChR by BrAcCh completely abolishes the specific IAS fluorescence enhancement and also, as expected from its occupancy of the binding sites, inhibits the high affinity binding of agonists such as ^{3}H-Carb.

Quantitation of the Extent of IAS Labelling

Since as shown above the specific IAS label which shows an enhancement on binding of ligands is competitive with bromoacetylcholine labelling, the extent of specific labelling under various conditions could be estimated from measurements of residual sites for ^{3}H-bromoacetylcholine.

The conditions used for IAS labelling, i.e., reduction and alkylation in Ca^{2+}-free Ringers at 4°C are based on those used by Moore & Raftery (73) for covalent incorporation of ^{3}H-BrAcCh into the receptor. However under these conditions the stoichiometry of ^{3}H-BrAcCh labelling was one for every two α-BuTx binding sites. More recently Wolosin et al. (74) have demonstrated that a second site can be labelled by ^{3}H-BrAcCh if the labelling conditions are changed either by increasing the temperature for alkylation to room temperature or by using a different buffer (15 mM Tris, 150 mM NaCl, 1.5 mM EDTA, 4.5 mM NaN_3, pH 7). Using the conditions given in Methods for IAS labelling and quantitation of residual ^{3}H-BrAcCh sites using procedures in which maximal ^{3}H-BrAcCh incorporation was two per receptor, IAS prelabelling consistently inhibited 40-60% of the sites for BrAcCh. The extent of IAS labelling could be increased in a qualitatively similar manner to ^{3}H-BrAcCh using the different alkylation conditions described by Wolosin et al. (74) but ^{3}H-BrAcCh labelling was in no case completely inhibited. By increasing the concentration of DTT in the reduction step from 50 μM to 300 μM the extent of specific IAS incorporation could also be increased but this consistently resulted in an apparent loss of high affinity binding sites for ^{3}H-Carb or ^{3}H-AcCh possibly due to a reduction in affinities for these ligands.

High Affinity Agonist Binding Monitored by IAS Fluorescence

The fluorescence of IAS-labelled AcChR is enhanced in a specific and saturable manner upon the addition of agonists (Figure 7). Despite the proximity of the probe to the high affinity agonist binding sites and its significant incorporation (~50% of sites for ^{3}H-BrAcCh) the probe does not perturb the equilibrium binding of agonists to these sites (71 and below). The IAS fluorescence does however reflect agonist binding and fluorescence titrations give dissociation constants which are in excellent agreement with those estimated from titration with radiolabelled ligands (71).

The fluorescence of bound IAS monitors binding of agonists to two high affinity sites per AcChR. Such a stoichiometry can be obtained from fluorescence titrations by agonist in which the AcChR concentration is much greater than the dissociation constant of the complex. Under these conditions (at sub-stoichiometric concentrations) virtually all the ligand will be bound by the AcChR with a proportional enhancement of fluorescence until saturation of the sites is reached. Fig. 8 compares such "stoichiometric" titrations of IAS labelled AcChR by ^{3}H-AcCh, monitoring either the IAS fluorescence enhancement or the concentration of bound ^{3}H-AcCh. In both cases saturation of binding was reached at a number of sites equivalent to the number of α-BuTx binding sites, i.e., 2 per AcChR. Therefore although only ~50% of the high affinity sites are modified by covalent attachment of an adjacent IAS molecule, it appears that the probe is an effective monitor of the whole receptor population.

Antagonist Binding to IAS-Labelled AcChR

There is considerable evidence both from affinity labelling (see 2) and competition experiments (75) that antagonists compete for the high affinity agonist

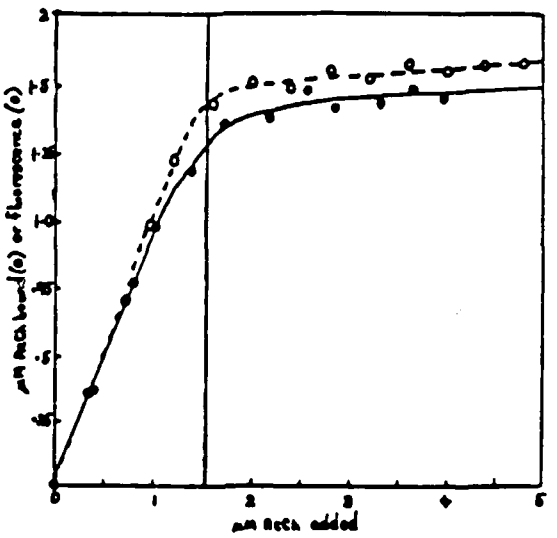


Figure 8.

binding sites on the α-subunits. It was therefore of interest to investigate whether antagonists also had an effect on the fluorescence of IAS attached near these sites.

The binding of both gallamine and hexamethonium to the IAS-labelled AcChR resulted in a saturable enhancement of IAS fluorescence allowing dissociation constants of 5.0±0.6 μM (gallamine) and 27±3 μM (hexamethonium) to be measured. Stopped-flow techniques have been used to measure complex formation between IAS-labelled membrane fragments and hexamethonium. The fluorescence enhancement at all hexamethonium concentrations could be fit with precision by a single exponential process and the rate constant had a hyperbolic dependence on concentration as shown in Fig. 9. These data are consistent with a mechanism in which a slow conformational change follows formation of the initial complex and that there is a rapid pre-equilibration between the bound and free receptorThe best-

$$R + L \underset{}{\overset{K_1}{\rightleftharpoons}} RL \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} R^*L$$

fit parameters for the hexamethonium binding data were 80.4 μM, 0.096 s^{-1} and 0.03 s^{-1} for K_1, k_2 and k_{-2} respectively. There is excellent agreement between the overall dissociation constant estimated from the kinetic data ($K_1 k_{-2}/k_2 = 25.1$ μM) and that obtained in equilibrium fluorescence titrations.

Investigation of the binding of tubocurarine to IAS-labelled AcChR was complicated by spectral overlap of tubocurarine with protein absorbance which

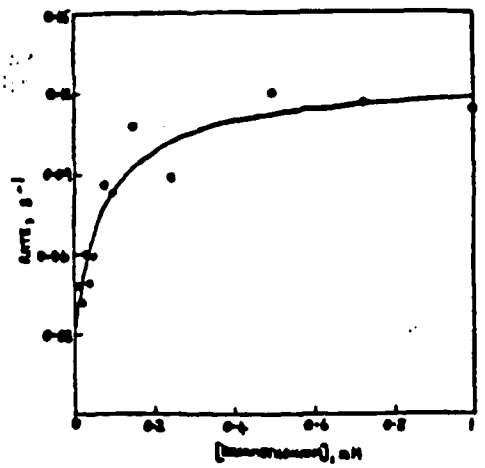


Figure 9.

restricted measurement of IAS fluorescence by energy transfer from the protein. However the equilibrium binding results suggested that binding was not to a homogeneous class of sites in agreement with the results of Neubig and Cohen (75) who showed that tubocurarine binding is characterized by two dissociation constants of 33 nM and 7.7 μM.

IANBD and IAS React at Different AcChR Locations

The specific fluorescence enhancement occurring on agonist or antagonist binding to IAS-labelled AcChR is due to IAS labelling of a reduced disulphide near the high affinity agonist binding sites on the α-subunits. Reaction of the AcChR by IANBD following mild reduction also results in a fluorescent receptor preparation whose fluorescence is enhanced on binding of agonists. There are several experimental observations which demonstrate that IANBD does not react in the same position as IAS: (1) the conditions used in the preliminary reduction to IANBD labelling are milder than those used for covalent incorporation of ^{3}H-BrAcCh or specific labelling by IAS, (2) the extent of specific IANBD labelling as estimated from its fluorescence enhancement on binding of agonists is unaffected by the presence of low (10-100 μM) concentrations of Carb during either the reduction or the alkylation, (3) pre-labelling of AcChR by covalently-bound BrAcCh does not affect the specific labelling by IANBD, (4) IANBD labelling, unlike IAS, does not significantly reduce the covalent incorporation of ^{3}H-BrAcCh, and (5) pre-labelling of the AcChR by IANBD does not inhibit specific incorporation of IAS. These results show that IANBD and IAS react with different sulfhydryl groups which is not surprising since IAS is a small, water soluble probe whereas IANBD is sparingly soluble in aqueous solution, and reacts and develops appreciable fluorescence only in a hydrophobic environment.

Double Labelling of the AcChR by IAS and IANBD

When membrane-bound AcChR was first labelled by IAS and then by IANBD the fluorescence emission spectra were characteristic of the incorporation of both fluorescent probes. IAS fluorescence may be excited directly at 340 nm or indirectly by energy transfer from the protein (Fig. 10) with emission at ~430 nm. IANBD

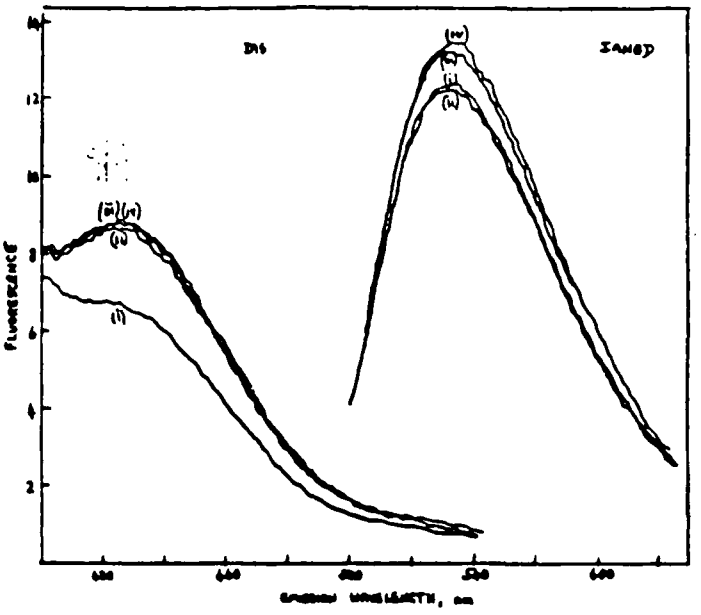


Figure 10.

absorbs at ~480 and fluoresces at 535 nm and therefore there is little spectral overlap between the two fluorophores. Addition of Carb to the doubly labelled preparation shows clearly that the enhancement of IAS fluorescence takes place at low concentrations and is saturable in the micromolar range. On the contrary micromolar concentrations of Carb have no effect on the fluorescence of covalently bound NBD and fluorescence enhancements of this probe occur only in the millimolar range. The two probes are therefore reflecting equilibrium binding to sites which have very different affinities.

Equilibrium Binding of Carbamylcholine to IANBD IAS Labelled AcChR

Labelling of AcChR by IAS, IANBD or both did not affect the dissociation constant for ^{3}H-Carb or the number of high affinity binding sites as shown for the doubly labelled preparation. Equilibrium fluorescence titrations in which the fluorescence of both probes was measured in parallel show that the estimated Kd obtained for Carb from the IAS fluorescence enhancement of ~0.207 μM (Fig. 11) is in excellent agreement with estimates from ^{3}H-Carb titrations. The Kd value

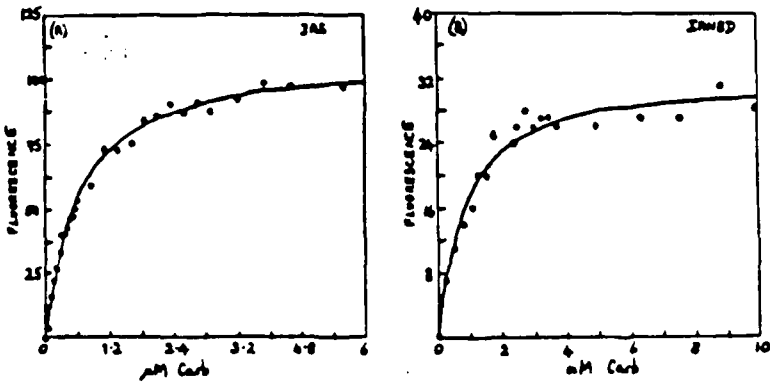


Figure 11.

obtained from the NBD fluorescence enhancement was 0.81 mM in agreement with previous results for low affinity agonist binding.

Kinetics of Agonist Binding to IANBD/IAS Labelled AcChR

The kinetics of the fluorescence enhancements occurring on agonist binding to IANBD/IAS labelled AcChR have been investigated in stopped-flow experiments. Over a range of carbamylcholine concentrations (0.1-10 mM) the NBD fluorescence enhancement occurred in a single fast phase as shown in Fig. 12. This is consistent

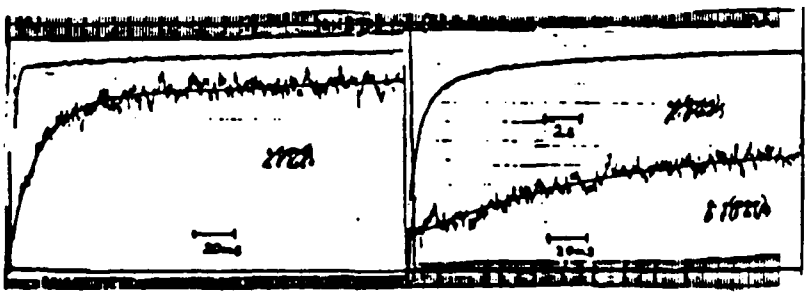


Figure 12.

with previous results in which we have shown that NBD reflects a fast conformational change of the receptor-agonist complex. In the same preparation at the same Carb concentrations the IAS fluorescence enhancement was multiphasic and the rate constants of each phase were slower than those observed with NBD no

concentration was there any agreement between the rates obtained from the fluorescence of the two probes (Fig. 12).

Double labelling of AcChR preparations by two sulfhydryl alkylating probes, IAS and IANBD has shown that the two fluorophores react in different locations and reflect agonist binding to different types of binding sites having very different affinities.

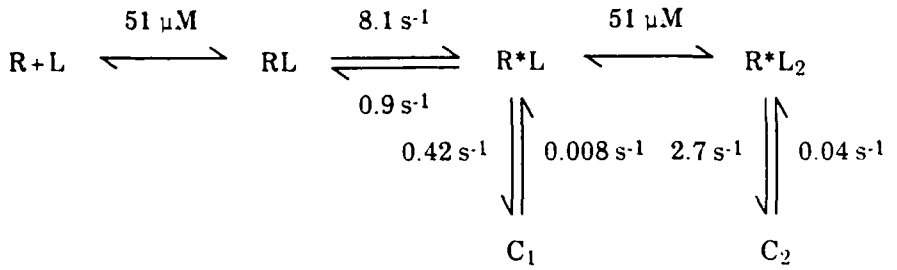
The specific fluorescence enhancement occurring on binding to IAS-labelled AcChR is due to labelling of cysteine residues generated by reduction of a reactive disulphide bond near high affinity binding sites on the α-subunits. Specific labelling is abolished by protection of this disulphide from reduction by inclusion of low concentrations of Carb (10 μM) at this step or by pre-labelling of the AcChR by the affinity reagent, ^{3}H-bromoacetylcholine. Quantitation of the extent of labelling by IAS from its inhibition of ^{3}H-BrAcCh sites has shown that routinely ~50% of the BrAcCh sites are inhibited. Covalent attachment of IAS in this position does not however perturb the equilibrium binding of agonists to their high affinity sites.

Labelling of the AcChR by IANBD also results in a significant incorporation of the fluorophore. We have previously shown that in a typical preparation approximately three IANBD molecular were incorporated per AcChR and these were associated with the α, β and γ subunits (62). IANBD does not compete for IAS or bromoacetylcholine labelling sites and therefore the fluorescence enhancement occurring on the binding of agonists is due to IANBD labelling in some part of the molecule other than the readily reduced disulfide on the α-subunits.

When AcChR preparations were labelled by both fluorophores, fluorescence titration data show clearly that the two probes are monitoring agonist binding to two classes of sites having different affinities. In the same AcChR sample, IAS revealed saturable binding to high affinity sites having a Kd of ~0.2 μM in agreement with the affinity of the unlabelled AcChR for ^{3}H-Carb. On the contrary, the fluorescence of IANBD was unaffected by low concentrations of Carb but showed a saturable enhancement with a Kd of ~1 mM, in excellent agreement with previous results and with the midpoint of the flux response (70,76).

The kinetics of the fluorescent enhancements of the two fluorophores in the same preparation were also quite different. At high Carb concentrations the IANBD fluorescence enhancement seemed to be monophasic and rapid ($k_{app} = 66\ s^{-1}$ at 1 mM Carb). The IAS fluorescence enhancement was more complicated and could be resolved into several phases all having rates significantly slower than those observed with IANBD. We have previously reported (71) a detailed analysis of the concentration dependence of Carb binding to IAS-labelled AcChR and the following model was proposed in which the pathway to C_1 corresponds to the high affinity binding measured in fluorescence titrations or in equilibrium binding of ^{3}H-Carb. All the conformational changes are two slow to be rate limiting steps in channel opening and also the dissociation constants are much lower than those measured in ion flux experiments. Desensitization has been shown in electrophysiological experiments to be characterized by several rate constants similar to those above and it is therefore likely that IAS reflects similar processes. Therefore binding to the high affinity sites does not display the characteristics of being involved in channel opening.

In the same preparations agonist induced changes in fluorescence of bound IANBD show clearly that the AcChR has a distinct class of low affinity agonist



binding sites which are present at equilibrium, i.e., in desensitized AcChR. The close agreement between dissociation constants obtained in fluorescence titrations and from ion flux measurements (70,76) is compelling evidence that these sites are functionally important in channel activation.

At the neuromuscular junction the concentration of acetylcholine soon after release is high (10^{-4}-10^{-3} M) and sufficient to occupy low affinity sites leading to a rapid conformational change and opening of the ion channel. At the same time the well characterized sites on the α-subunits will be occupied since the bimolecular association of ligand with all its available sites is likely to be close to diffusion control. Eventually as a result of slow conformational change these α-subunit sites have a high affinity for agonists. However, the distribution of resting state, open and closed/desensitized states is kinetically determined by the relative affinities of the binding sites and the rates of the conformational changes associated with each binding event. *In vivo* the slow transitions leading to desensitization are unlikely to occur due tot he short time of occupancy of the sites as a consequence of rapid diffusion of acetylcholine out of the cleft and its hydrolysis by acetylcholinesterase. Control of channel opening by neurotransmitter binding to low affinity sites allows rapid equilibration between resting and open states and allows for rapid closing of the channel as the concentration of acetylcholine declines.

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 (109). Further purification is achieved by base treatment (20,110) and intact vesicles separated from membrane pieces and leaky vesicles by rapid centrifugation in 8-20% sucrose gradients (45).

2. Exposure of acetylcholine receptor to the lipid bilayer

Membrane fragments enriched in AChR were prepared from electric organs from T. californica as described by Elliott et al. (80) and were further purified by alkali extraction, which removes peripheral proteins (81,82). The membranes were finally suspended in Torpedo Ringer's solution adjusted to pH 7.4 (250 mM NaCl, 5 mM KCl, 20 mM Hepes, 0.02% NaN_3, and 2 mM $MgCl_2$). Protein concentration was determined by the method of Lowry et al. (83) The concentration of α-BgTx sites was determined by the method of Schmidt and Raftery (84) using [^{125}I] α-BgTx obtained from New England Nuclear and calibrated by the procedures of Blanchard et al. (85). The specific activity of the preparations varied between 1.4 and 3.6 nmol of α-BgTx binding sites/mg of protein. All tritium samples were counted on a Beckman LS 233 counter following addition of 10 ml of Aquasol 2 (New England Nuclear).

Conversion of the primarily 13S dimeric form of AChR obtained in the membrane fragments to the 9S monomeric form was accomplished using the method of Conti-Tronconi et al. (86). Reduction and alkylation were performed in Torpedo Ringer's solution adjusted to pH 8.5. After centrifugation, the membranes were resuspended in Torpedo Ringer's solution (pH 7.4). The extent of the reaction was determined by sucrose gradient centrifugation of solubilized receptors trace labeled with [^{125}I] α-BgTx. Typically, 99% of α-BgTx binding activity sediments in the 9S monomeric form after reduction and alkylation.

Synthesis of [^{3}H]Adamantanediazirine: [^{3}H]Adamantanediazirine (596 mCi/mmol) was synthesized and purified by the method of Bayley and Knowles (38). [^{3}H]-Adamantanone ethylene ketal needed for the synthesis was prepared by Amersham Corporation from bromoadamantanone ethylene ketal, which we provided.

[^{3}H]Adamantanediazirine Binding to Membrane Fragments: [^{3}H]Adamantanediazirine (9 mCi/mmol) in ethanol was added to membrane fragments in Torpedo Ringer's solution at room temperature under dim light. In each of 4 samples, the final concentrations of [^{3}H]adamantanediazirine, membrane protein, and ethanol were 25 μM, 1 mg/ml, and 1% respectively. After 1 h incubation in the dark, the radioactivity in a portion of each sample was determined. The samples were then centrifuged at 147,000 g for 45 min. The radioactivity of the supernatant and the resuspended pellets was then determined. No correction was made for trapped volume of free solution in the pellet. The radioactivity in the pellet was interpreted as the amount of ligand bound to membrane fragments. Counting efficiency was accounted for using [^{3}H]H_2O as an internal standard.

Labeling of Membrane Fragments: All photolabeling experiments were carried out in Torpedo Ringer's solution with constant stirring at room temperature. [^{3}H]Adamantanediazirine (596 mCi/mmol) in ethanol was added to membrane fragments in a quartz cuvette under dim light. The final concentrations of [^{3}H]adamantanediazirine, membrane protein, and ethanol were 33 μM, 1 mg/ml, and 1% respectively. The sample was incubated in the dark for 30 min and then irradiated for 30 min using a UVSL-25 lamp (Ultraviolet Products) on the long wavelength setting. Preliminary experiments revealed that 30 min was sufficient for complete photolysis of the diazirine. After photolysis, the membrane fragments were diluted 28 fold with 1% BSA in Torpedo Ringer's solution. Following a 15 min incubation, a portion was removed to determine the radioactivity and the sample was then centrifuged for 15 min at 252,000 g. The BSA wash of the pellet was repeated, followed by 2 washes in buffer without BSA. Portions of both the supernatant and pellet were taken for determination of the radioactivity. The radioactivity remaining in the pellet was considered to represent the covalent attachment of radiolabel to membrane fragments.

Gel Electrophoresis: $NaDodSO_4$-polyacrylamide gel electrophoresis was performed using the system of Laemmli (87) with 8.75% (wt/vol) acrylamide cylindrical gels (0.5 x 10 cm). After electrophoresis, gels were stained with 0.5% Coomassie Blue in 10% acetic acid/25% propanol/65% water and destained in the same solvent without the dye. Gels were then sliced with a Hoeffer Scientific Instruments gel slicer. Each slice was sealed in a glass scintillation vial with 0.5 ml H_2O_2 and incubated at 60°C for 5 hours followed by determination of radioactivity.

Preparative gel electrophoresis and electroelution of the purified subunits from the gel was carried out using the method of Conti-Tronconi et al. (88). Amino acid analysis was used to quantitate the purified subunits in a portion of the sample. The subunits were hydrolysed in vacuo with 6N HCl at 110°C for 24 h. The hydrosylates were dried under vacuum and analysed according to Spackman (89) on a Beckman Model 120B amino acid analyser. After determination of the radioactivity in an identical portion of the sample, the specific incorporation of radiolabel into each subunit was calculated. Quenching was accounted for using [^{3}H]H_2O as an internal standard.

3. Labeling of Membrane Fragments

All photolabeling experiments were carried out in *Torpedo* Ringer's solution with constant stirring at room temperature. [3H]Adamantanediazirine (596 mCi/mmol) in ethanol was added to membrane fragments in a quartz cuvette under dim light. The final concentrations of [3H]adamantanediazirine, membrane protein, and ethanol were 33 μM, 1 mg/ml, and 1% respectively. The sample was incubated in the dark for 30 min and then irradiated for 30 min using a UVSL-25 lamp (Ultraviolet Product) on the long wavelength setting. Preliminary experiments revealed that 30 min was sufficient for complete photolysis of the diazirine. After photolysis, the membrane fragments were diluated 28 fold with 1% BSA in *Torpedo* Ringer's solution. Following a 15 min incubation, a portion was removed to determine the radioactivity and the sample was then centrifuged for 15 min at 252,000 g. The BSA wash of the pellet was repeated, followed by 2 washes in buffer without BSA. Portions of both the supernatant and pellet were taken for determination of the radioactivity. The radioactivity remaining in the pellet was considered to represent the covalent attachment of radiolabeled to membrane fragments.

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