Cholinergic Binding Sites on the Pentameric Acetylcholine Receptor of *Torpedo californica*†

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ABSTRACT: The binding of agonists, antagonists, and the acetylcholinesterase inhibitor, eserine, to the nicotinic acetylcholine receptor from *Torpedo californica* has been monitored by the fluorescence changes of two extrinsic probes that have been covalently attached to the receptor protein. Although both probes, 5-(iodoacetamido)salicylic acid (IAS) and 4-[*N*-[(2-iodoacetoxy)ethyl]-*N*-methylamino]-7-nitrobenz-2-oxa-1,3-diazole (IANBD) react with sulfhydryl groups, they do not react at the same location. The conditions for IAS labeling and competition studies have shown that, following reduction of the receptor, this fluorophore reacts with the same cysteines on each of the two α subunits that may be labeled by the alkylating agonist, [^{3}H]bromoacetylcholine. The fluorescence of this probe is sensitive to the binding of agonists and competitive antagonists to two high-affinity sites on the receptor. IANBD does not react with the same cysteines as IAS, and its fluorescence is unchanged by the high-affinity binding of agonists and antagonists. The fluorescence of this probe is, however, specifically and saturably enhanced by the binding of agonists to distinct low-affinity sites. Heterogeneity in the NBD fluorescence changes induced by the bis-quaternary agonist, suberyldicholine, has indicated that the stoichiometry of low-affinity sites is also two per receptor. Stopped-flow studies of agonist binding to receptor preparations that had been doubly labeled by both fluorophores demonstrate that the conformational changes detected by IAS occur on slow time scales of seconds to minutes whereas a much faster conformational change is revealed by changes in NBD fluorescence. These results provide further evidence that the acetylcholine receptor carries two distinct classes of sites for classical agonists and are consistent with the involvement of the low-affinity sites in channel activation. Eserine, which has been reported to activate the acetylcholine receptor channel at a concentration of 50–100 μM via a pathway that is not inhibited by α-bungarotoxin [Okonjo, K. O., Kuhlmann, J., & Maelicke, A. (1991) *Eur. J. Biochem. 200*, 671–677], also induces a saturable enhancement of NBD fluorescence (K_d approximately 50 μM) that is not blocked by α-bungarotoxin. These results are consistent with the notion that the NBD fluorescence changes induced by high concentrations of agonists are a reflection of the induction of cation translocation.

Fluorescence methods have been widely used to measure the interaction of ligands with the nicotinic acetylcholine receptor (nAChR),[1] in both its soluble and membrane-bound states. These methods include the monitoring of protein intrinsic fluorescence, the binding of fluorescent ligands, and measurements of conformational changes using extrinsic probes either reversibly or covalently bound to the receptor protein [reviewed by Conti-Tronconi and Raftery, (1982) and Ochoa et al. (1989)]. The major aim in the use of these techniques has been to develop kinetic methods for monitoring conformational changes of the receptor protein that may be important in its functional responses of channel activation and receptor desensitization.

The nAcChR is a pentameric complex of four homologous subunits in the ratio $\alpha_2\beta\gamma\delta$ (Raftery et al., 1980; Noda et al., 1983). Equilibrium binding measurements have shown that each nAcChR carries two high-affinity binding sites for agonists and competitive antagonists. The results of affinity labeling studies have shown that these sites are located, at least in part, on the α subunits. Following reduction of a reactive disulfide bond formed by adjacent cysteines at positions 192 and 193 in the α subunits (Kao & Karlin, 1986; Mosckovitz & Gershoni, 1988; Kellaris & Ware, 1989), these cysteines may be labeled by the alkylating antagonist, [^{3}H]-[4-(*N*-maleimido)benzyl]trimethylammonium (MBTA; Kao et al., 1984). The alkylating agonist, [^{3}H]bromoacetylcholine, also labels each of the two α subunits of the reduced receptor, presumably also as a result of reaction with Cys-192 and Cys-193 (Damle et al., 1978; Moore & Raftery, 1979a; Wolosin et al., 1980). It was recently suggested that these sites are not associated exclusively with the α subunits but are located at the α–γ and α–δ subunit interfaces (Blount & Merlie, 1989). This suggestion has been supported by recent photoaffinity labeling studies. Labeling with [^{3}H]tubocurarine resulted in covalent incorporation not only into α, but also into the γ and δ subunits (Pederson & Cohen, 1990), and [^{3}H]nicotine photoaffinity labeled both α and δ subunits, with most of the

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[1] Abbreviations: AcCh, acetylcholine; α-BuTx, α-bungarotoxin; BrAcCh, bromoacetylcholine; Carb, carbamylcholine; DEAE, diethylaminoethyl; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; GABA, γ-aminobutyric acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; 5-HT, 5-hydroxytryptamine; IANBD, 4-[*N*-[2-(iodoacetoxy)ethyl]-*N*-methylamino]-7-nitrobenz-2-oxa-1,3-diazole; IAS, 5-(iodoacetamido)salicylic acid; MBTA, [4-(*N*-maleimido)-benzyl]trimethylammonium diiodide; nAcChR, nicotinic acetylcholine receptor; NBD, 4-[*N*-[(acetoxy)ethyl]-*N*-methylamino]-7-nitrobenz-2-oxa-1,3-diazole; SdCh, suberyldicholine; Tris, tris(hydroxymethyl)-aminomethane.

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α subunit label being incorporated into Tyr-198 (Middleton & Cohen, 1991).

In most of the models that have been proposed to describe the pathways leading from agonist binding to the opening of the ion channel and receptor desensitization, it has been assumed that the two sites identified in labeling studies are the only agonist binding sites on the nAcChR [reviewed by Conti-Tronconi and Raftery (1982), Ochoa et al. (1989), and Stroud et al. (1990)]. However, we have previously described the presence of additional low-affinity sites for agonists on the *Torpedo* nAcChR (Conti-Tronconi et al., 1982; Dunn & Raftery, 1982a,b; Dunn et al., 1983; Raftery et al., 1983). These sites were revealed by the fluorescence changes of a probe, IANBD, which was covalently bound to the receptor on three (α, β, γ) of the four subunits (Conti-Tronconi et al., 1982). Agonist binding to NBD-labeled receptor preparations was accompanied by a saturable enhancement of NBD fluorescence, which was inhibited by α-bungarotoxin but unaffected by other cholinergic antagonists and local anesthetics (Dunn & Raftery, 1982a,b). The characteristics of agonist binding to the low-affinity sites, particularly the excellent agreement between dissociation constants estimated from fluorescence titrations of NBD-labeled receptor and from cation flux responses, in addition to the rapidity of the conformational change that results from binding, led us to propose that occupancy of these sites may be important for channel activation (Raftery et al., 1983). In the case of acetylcholine (AcCh), the estimated K_d values of approximately 100 μM are in agreement with electrophysiological measurements of dose–responses at the neuromuscular junction (Adams, 1981). Furthermore, such low-affinity binding is consistent with the high local concentrations of AcCh (approaching millimolar) that are estimated to be present in the synaptic cleft soon after neurotransmitter release (Hartzell et al., 1975; Lester et al., 1978; Land et al., 1980). In contrast to the rapid conformational changes accompanying agonist binding to the low-affinity sites, the conformational changes associated with binding to the high-affinity sites associated with the α subunits (and likely also with the γ and δ subunits) are considerably slower (see below), and we have proposed that these may reflect pathways leading to channel closing and desensitization (Raftery et al., 1983).

The likelihood of multiple agonist binding sites being present on some or all of the subunits is supported by the sequence homology of the subunits (Raftery et al., 1980; Noda et al., 1983), which first suggested the possibility of homologous binding domains. Such sequencing approaches were used to first demonstrate the existence of a family of related nicotinic AcChRs in the peripheral and central nervous systems (Stroud et al., 1990). More recently, it has been shown that the sequence homology of nAcChR subunits extends to other members of the ligand-gated ion channel family, including the $GABA_A$ (Schofield et al., 1987), glycine (Grenningloh et al., 1987), and $5\text{-}HT_3$ receptors (Maricq et al., 1991). Direct evidence that each of the $GABA_A$ receptor subunits carries a binding site (or sites) for GABA and other GABAergic ligands has come from the observation that individual subunits of this receptor can form homo-oligomeric GABA-gated chloride channels when expressed in *Xenopus* oocytes from their encoding mRNAs (Blair et al., 1988). Similar expression of homo-oligomeric channels has been reported for subunits of the glycine [reviewed by Betz (1991)] and $5HT_3$ (Maricq et al., 1991) receptors, suggesting that multiplicity of agonist binding sites may be a common feature of all subunits in this channel "superfamily". In view of the significance of these binding sites in the receptor mechanism, it is clearly important that agonist binding be further characterized.

In this report we describe the double labeling of the nAcChR by two fluorescent probes, one of which, IAS, has been used previously in studies of agonist binding (Dunn et al., 1980, 1981; Blanchard et al., 1982) and which monitors binding to the high-affinity sites located, at least in part, on the α subunits, and the other, IANBD, reflects low-affinity binding as described above. These studies are extended to the investigation of eserine, a reversible acetylcholinesterase inhibitor, which has recently been reported to induce cation flux by the nAcChR in muscle (Pascuzzo et al., 1984) and, in the case of *Torpedo* nAcChR, to induce cation flux even in the presence of saturating concentrations of the competitive antagonists of AcCh binding, tubocurarine, and α-BuTx (Okonjo et al., 1991). It is shown that both high- and low-affinity sites for agonists exist in the same preparations. Furthermore, eserine induces a fluorescence enhancement of NBD-labeled nAcChR, similar to the behavior of other receptor activators, and this enhancement is not inhibited by α-BuTx. These results suggest a pathway(s) for activation of nAcChR that does not depend on conformational transitions of the high-affinity sites even though these sites are most likely to be occupied by agonists during the activation process.

MATERIALS AND METHODS

Preparation and nAcChR-Enriched Membrane Fragments. nAcChR-enriched membrane fractions were prepared from *Torpedo californica* electric organ and were alkali-extracted to remove nonreceptor protein as previously described (Elliott et al., 1980), with the exception that, for experiments involving IANBD, iodoacetamide was excluded from the initial homogenization. Specific activities lay in the range of 2–4 nmol of [^{125}I]-α-BuTx (New England Nuclear) sites per milligram of protein. Unless otherwise stated, the buffer used in each experiment was *Torpedo* Ringers (20 mM Hepes, pH 7.4, 250 mM NaCl, 5 mM KCl, 4 mM $CaCl_2$, 2 mM $MgCl_2$, 0.02% NaN_3).

Labeling of Membrane Fragments by 5-IAS. Membranes were diluted to 1 μM in [^{125}I]-α-BuTx sites in Ca^{2+}-free Ringers and were reduced with 50 μM DTT for 60 min at 4 °C. 5-IAS (Molecular Probes Inc., Eugene, OR) was added to a final concentration of 250 μM, and the mixture was stirred for 2 h at 4 °C, after which the membranes were collected by centrifugation and washed to remove unreacted reagents (Dunn et al., 1980).

Labeling of Membranes by IANBD. Labeling of nAcChR-enriched membranes by IANBD was carried out as described previously (Dunn & Raftery, 1982b). Membranes were diluted to 10 μM in [^{125}I]-α-BuTx sites in 10 mM Hepes, pH 7.4, and 35 mM $NaNO_3$ and reduced with 50 μM DTT for 15 min at room temperature. Solid IANBD was added as a finely ground powder to a nominal concentration of 300 μM, and the membranes were stirred for 2 h at 4 °C. Unreacted reagent and residual solid IANBD were removed by gel filtration on a Sephadex G-25-300 (Sigma Chemical Co., St. Louis, MO) column equilibrated in Ca^{2+}-free Ringers.

Double Labeling of nAcChR by 5-IAS and IANBD. Double labeling of nAcChR by the two fluorescent probes was achieved by first labeling with IANBD, concentrating the membranes by centrifugation, and then carrying out the IAS labeling procedures as described above.

Affinity Labeling with [^{3}H]Bromoacetylcholine. [^{3}H]-Bromoacetylcholine (BrAcCh) was prepared by the reaction of [^{3}H]choline (New England Nuclear) with bromoacetyl bromide (Aldrich) as described (Moore & Raftery, 1979a).

Labeling of nAcChR by [^{3}H]bromoacetylcholine was as described by Wolosin et al. (1980) with modifications (Dunn et al., 1983). Under an argon atmosphere, membrane fragments were diluted to 1 μM in [^{125}I]-α-BuTx sites in 15 mM Tris HCl, pH 8.0, 150 mM NaCl, 4.5 mM NaN_3, and 1.5 mM EDTA and reduced with 300 μM DTT for 45 min at room temperature. The membranes were centrifuged to remove DTT and resuspended in the same buffer but at pH 7.0 and reacted with 40 μM [^{3}H]BrAcCh for 30 min. The extent of labeling was estimated by DEAE–disc assay (Moore & Raftery, 1979a) or by $NaDodSO_4$ gel electrophoresis (Laemmli, 1970) followed by cutting of 2-mm strips, digestion in 30% hydrogen peroxide, and counting for [^{3}H].

Equilibrium Fluorescence Measurements. Equilibrium fluorescence measurements were made using a Perkin-Elmer MPF4 fluorometer and appropriate monitoring conditions for IAS fluorescence (via energy transfer from the protein) and NBD fluorescence. Since IAS monitors high-affinity binding and there is significant depletion of added ligand due to binding, fluorescence titration data were fit by nonlinear regression methods (Dunn et al., 1980) by the equation:

$$Fl = 0.5 \times F_0[(L_0 + R_0 + K_d) - \{(L_0 + R_0 + K_d)^2 - 4R_0L_0\}^{1/2}]$$

where Fl is the observed fluorescence (after subtraction of background due to IAS-labeled membranes alone), L_0 is the total added ligand concentration and the unknown parameters, and R_0, K_d, and F_0 are the concentration of binding sites, the dissociation constant, and the maximum fluorescence enhancement, respectively.

Titrations of NBD-labeled nAcChR, since they reveal low-affinity binding sites and there is no significant depletion of added ligand, were fit by the simple binding model:

$$Fl = F_0L_0/(K_d + L_0)$$

where the parameters are as defined above.

Stopped-Flow Fluorescence Experiments. Kinetic data were obtained using a Durrum D-110 stopped-flow instrument and the data collection and analysis procedures previously described (Dunn et al., 1980), using the appropriate monitoring conditions for IAS fluorescence excited via energy transfer (Dunn et al., 1980) or IANBD fluorescence (Dunn & Raftery, 1982b).

Binding of Radiolabeled Agonist. [^{3}H]AcCh and [^{3}H]-carbamylcholine (Carb) binding to membrane-bound nAcChR was measured using the centrifugation assay previously described (Dunn et al., 1980).

RESULTS

Localization of the IAS Label. Following mild reduction of nAcChR, alkylation by 5-IAS gives rise to a highly fluorescent receptor preparation whose fluorescence is enhanced in a specific and saturable manner on the binding of agonists (Figure 1) or competitive antagonists (see below). As discussed above, each α subunit carries a reactive disulfide bond formed by vicinal cysteines 192 and 193, and, after reduction, these residues are thought to be the sites of covalent incorporation of [^{3}H]BrAcCh. We previously reported that prelabeling of nAcChR by IAS partially inhibited the covalent incorporation of [^{3}H]BrAcCh (Dunn et al., 1980). Although, in the earlier study, the extent of inhibition was not quantitated, we suggested that IAS reacts with the same sulfhydryl groups as [^{3}H]BrAcCh. The results in Figure 1 show that the fluorescence enhancement occurring on the binding of ligands

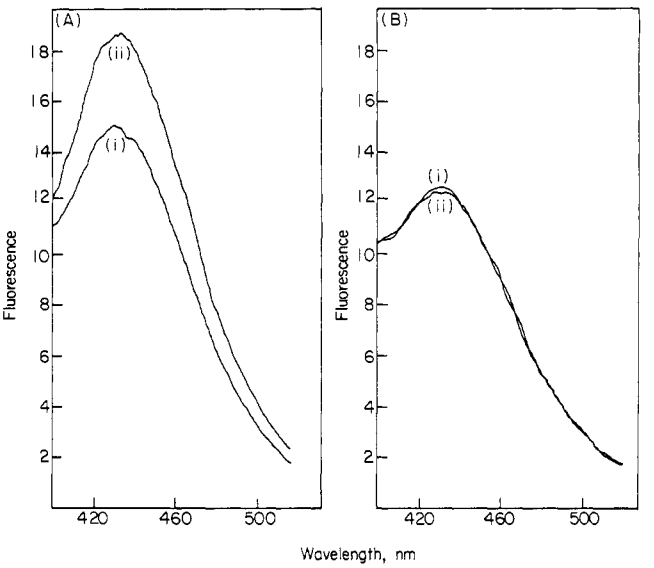


FIGURE 1: Effect of the presence of 10 μM Carb during DTT reduction on the agonist-induced fluorescence enhancement of IAS-labeled nAcChR. Membrane fragments were reduced either in the absence (A) or presence (B) of 10 μM Carb. The agonist was then removed by centrifugation and the membranes were labeled by IAS as described in the Materials and Methods. Fluorescence spectra of nAcChR (0.6 μM in α-BuTx sites) alone (i) or after incubation with 50 μM Carb (ii) were recorded using an excitation wavelength of 290 nm.

to IAS-labeled nAcChR is due specifically to the probe bound in this position. When the reactive disulfides near the high-affinity sites were protected from reduction by the presence of low concentrations of Carb during the reaction with DTT (Bregestovski et al., 1977; Damle & Karlin, 1980), the fluorescence enhancement occurring on ligand binding was completely abolished, although there was still a significant nonspecific incorporation of IAS. If Carb was included at the alkylation step but not during the reduction, there was a reduced but still significant enhancement of fluorescence upon agonist binding. Thus occupancy of the high-affinity sites by agonists during reduction and, to a lesser extent, during the long alkylation procedures inhibits IAS labeling of those sulfhydryl groups that give rise to the specific fluorescence enhancement. Similar experiments have previously been used to demonstrate the specificity of [^{3}H]BrAcCh labeling on the α subunits (Bregestovski et al., 1977). In control experiments, labeling of nAcChR by BrAcCh prior to IAS labeling has been shown to completely abolish all specific IAS labeling. Thus BrAcCh and IAS compete for the same sulfhydryl groups near the high-affinity binding sites, and the fluorescent enhancement observed upon ligand binding to IAS-labeled nAcChR is likely to be due to IAS labeling of Cys-192 and/or Cys-193. In view of the proximity of the IAS label to the high-affinity sites, it is important to note that its incorporation 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 cation flux responses (Dunn et al., 1980).

Quantitation of the Extent of IAS Labeling. Since, as described above, the specific IAS label which yields the fluorescence enhancement upon ligand binding is competitive with bromoacetylcholine labeling, the extent of specific labeling could be estimated from measurements of the number of residual sites for [^{3}H]BrAcCh. The conditions used for IAS labeling, i.e., reduction and alkylation in Ca^{2+}-free Ringers at 4 °C, were based on those used by Moore and Raftery (1979a) for covalent labeling of the receptor by [^{3}H]BrAcCh. These conditions lead to labeling of only one of the two α

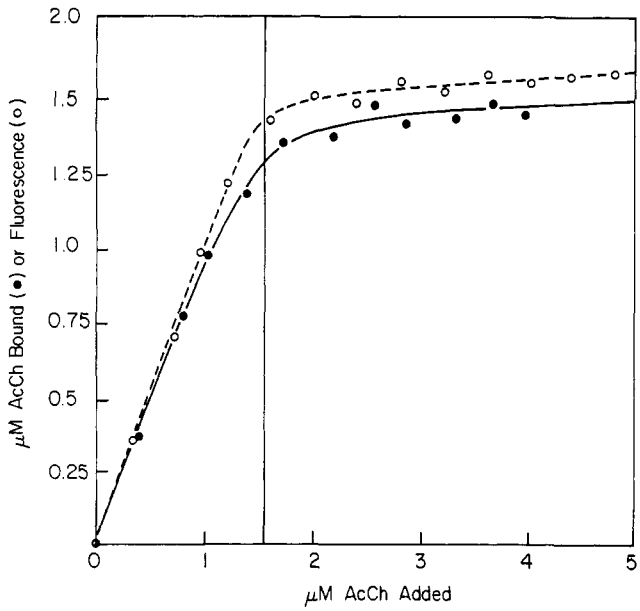


FIGURE 2: Titration of IAS-labeled nAcChR (1.5 μM in α-BuTx sites) by [^{3}H]AcCh, monitoring either the enhancement of IAS fluorescence using excitation and emission wavelengths of 290 and 340 nm, respectively (O), or bound radioactivity (●).

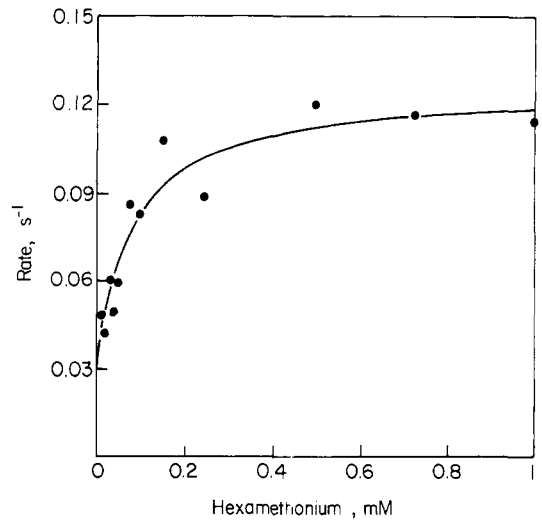


FIGURE 3: Kinetics of hexamethonium binding to IAS-labeled nAcChR (0.5 μM in α-BuTx sites). Apparent rate constants were obtained in stopped-flow experiments and fitting of the reaction traces by a single-exponential model. Data were fit by a model in which a conformation change follows the formation of the initial complex as described in the text. The solid line is calculated using the best fit parameters K_1 = 80.4 μM, k_2 = 0.096 s^{-1}, and k_2 = 0.03 s^{-1}.

subunits by [^{3}H]BrAcCh, but Wolosin et al. (1980) later established conditions to label both of the available sites. Using the former conditions, IAS prelabeling consistently inhibited 40–60% of the available sites for [^{3}H]BrAcCh when these were measured by the procedures of Wolosin et al. (1980). The extent of IAS labeling could be increased such that 80% of the [^{3}HBrAcCh sites were inhibited by using the alkylation conditions of Wolosin et al. (1980), although in no case was [^{3}H]BrAcCh labeling completely abolished. By increasing the concentration of DTT in the reduction step to 300 μM, the extent of IAS labeling could also be increased, but this consistently led to an apparent loss of high-affinity sites for [^{3}H]Carb and [^{3}H]AcCh. Extensive reduction and alkylation of nAcChR has previously been reported to cause a decrease in agonist affinity (Moore & Raftery, 1979b; Walker et al., 1981; Blanchard et al., 1982), and the observed loss of sites is likely to be due to reduction of a receptor disulfide other than that at Cys-192/193.

High-Affinity Binding of Agonists Monitored by Changes in IAS Fluorescence. Despite the proximity of the IAS label to the high-affinity agonist sites and its significant level of incorporation (40–60% of available sites), the probe does not perturb the equilibrium binding of agonists to these sites and K_d values obtained from fluorescence titrations are in good agreement with values obtained from measurements of radiolabeled ligand binding (Dunn et al., 1980; Blanchard et al., 1982). The fluorescence of bound IAS monitors the binding of agonists to two high-affinity sites per nAcChR. Such a stoichiometry can be obtained from fluorescence titrations by agonist using an nAcChR concentration which is much greater than the dissociation constant of the complex. Under these conditions, at substoichiometric concentrations of ligand, virtually all ligand will be bound with a proportional enhancement of fluorescence until saturation of binding sites is reached. Figure 2 compares such "stoichiometric" titrations of IAS-labeled nAcChR by [^{3}H]AcCh, monitoring either the fluorescence enhancement or the concentration of bound radiolabel. In both cases, saturation was reached at a number of sites equivalent to the number of sites for [^{125}I]-α-BuTx, i.e., two per nAcChR. Therefore, although IAS routinely labels only approximately 50% of the available sites, the probe appears to be an effective monitor of all high-affinity sites in the receptor population.

Antagonist Binding to IAS-Labeled nAcChR. There is considerable evidence both from affinity labeling studies (Damle et al., 1978) and from competition experiments (Neubig & Cohen, 1979) that agonists and antagonists compete for the high-affinity sites associated with the α subunits [reviewed by McCarthy et al. (1986)]. Since the binding of agonists and that of antagonists induce different conformational states of nAcChR, it was of interest to investigate whether competitive antagonists also have an effect on the fluorescence of IAS covalently attached close to the sites. The binding of both gallamine and hexamethonium to the IAS-labeled nAcChR resulted in a saturable enhancement of IAS fluorescence, allowing dissociation constants of 5.0 ± 0.6 μM and 27 ± 3 μM to be measured for gallamine and hexamethonium, respectively. The mechanism of binding of hexamethonium has been investigated in stopped-flow experiments. At all hexamethonium concentrations, the fluorescence enhancement could be fit with precision by a single-exponential model and the apparent rate had a hyperbolic dependence on ligand concentration as shown in Figure 3. These data are consistent with a mechanism in which a slow conformational change follows formation of the initial complex and in which there is a rapid equilibration between free and bound receptor:

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

The best 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 thus reasonable agreement between the overall dissociation constant estimated from the kinetic data [$K_1K_2/(1 + K_2)$ = 19.1 μM] and that estimated from equilibrium fluorescence titrations (27 ± 3 μM).

Investigation of tubocurarine binding to IAS-labeled nAcChR was complicated by spectral overlap between tubocurarine and protein absorbance. The IAS fluorescence could not, therefore, be measured by energy transfer, necessitating the use of direct excitation, which is considerably less sensitive (Dunn et al., 1980). The binding kinetics were biphasic and the equilibrium results suggested that binding was not to a

homogeneous class of sites. This evidence for heterogeneity is consistent with earlier results of Neubig and Cohen (1979), who showed that tubocurarine binding is characterized by two dissociation constants of 33 nM and 7.7 μM.

IANBD and IAS React at Different nAcChR Location. As shown above, the fluorescence enhancement occurring on agonist or antagonist binding to IAS-labeled receptor preparations is due specifically to IAS labeling of a reduced disulfide on the α subunits. Reaction of nAcChR with IANBD following mild reduction (50 μM DTT, 15 min at room temperature) also results in a fluorescent receptor preparation whose fluorescence is enhanced by the binding of agonists, although much higher concentrations of agonists are required than those that affect IAS fluorescence (Dunn & Raftery, 1982a,b). There are several experimental observations that demonstrate that IANBD does not react in the same position as IAS: (a) the extent of specific IANBD labeling as estimated by the magnitude of the fluorescence enhancement occurring upon agonist binding is unaffected by low (10–100 μM) concentrations of Carb during either the reduction or alkylation; (b) prelabeling of nAcChR by covalent reaction with BrAcCh does not affect the specific labeling by IANBD; (c) IANBD labeling, in contrast to IAS, does not reduce the extent of subsequent incorporation of [^{3}H]BrAcCh; (d) prelabeling of nAcChR by IANBD does not inhibit the specific incorporation of IAS. Thus, the fluorescence changes occuring upon binding of agonists to IAS- or NBD-labeled preparations arise from labeling of distinct sulfhydryl groups of the receptor, which is not surprising since IAS is a small water-soluble fluorophore whereas IANBD is only sparingly soluble in aqueous solution and reacts and develops appreciable fluorescence only in a hydrophobic environment (Haugland, 1975).

Stoichiometry of Low-Affinity Sites Revealed by NBD Fluorescence. Evidence that NBD fluorescence monitors the binding of agonists to two low-affinity sites on the nAcChR has been obtained from studying the binding of the bisquaternary agonist, suberyldicholine (SdCh). In initial studies, measurements of SdCh binding to NBD-labeled preparations were hampered by the low amplitude of the fluorescence signal change, which was consistently only about 50% that induced by AcCh or Carb. The results illustrated in Figure 4A provide an explanation for this low amplitude and show that, whereas SdCh binds to one low-affinity site with a K_d of about 2 μM, Carb binds to two low-affinity sites with apparently equal affinity. When NBD-labeled nAcChR was titrated by SdCh until well beyond saturation of this binding site ([SdCh] = 100 μM) and the titration was continued by Carb, a second site was revealed with a K_d similar to that obtained in the absence of SdCh, but with only half of the amplitude change. Similar results have been obtained using AcCh as the second agonist. A more detailed examination of the binding of SdCh to NBD-labeled nAcChR has revealed that, for this agonist, there is also a second site but this has much lower affinity, with a K_d of about 2.5 mM (Figure 4B). Thus the low-affinity binding of Carb and AcCh appears to be to two sites having similar affinity, whereas the low-affinity sites for SdCh have much different affinities. In the case of SdCh, it would appear that only the site of higher affinity ($K_d \sim 2$ μM) must be occupied for channel activation since the midpoint of the flux response occurs at 1–3 μM (unpublished results).

Double Labeling of nAcChR by IAS and IANBD. When membrane-bound nAcChR was first labeled by IANBD and then by IAS, the fluorescence spectra were characteristic of the incorporation of both fluorescent probes as shown in Figure 5. In the doubly labeled preparation, the extent of incorporation of each probe, as estimated from the incorporation of

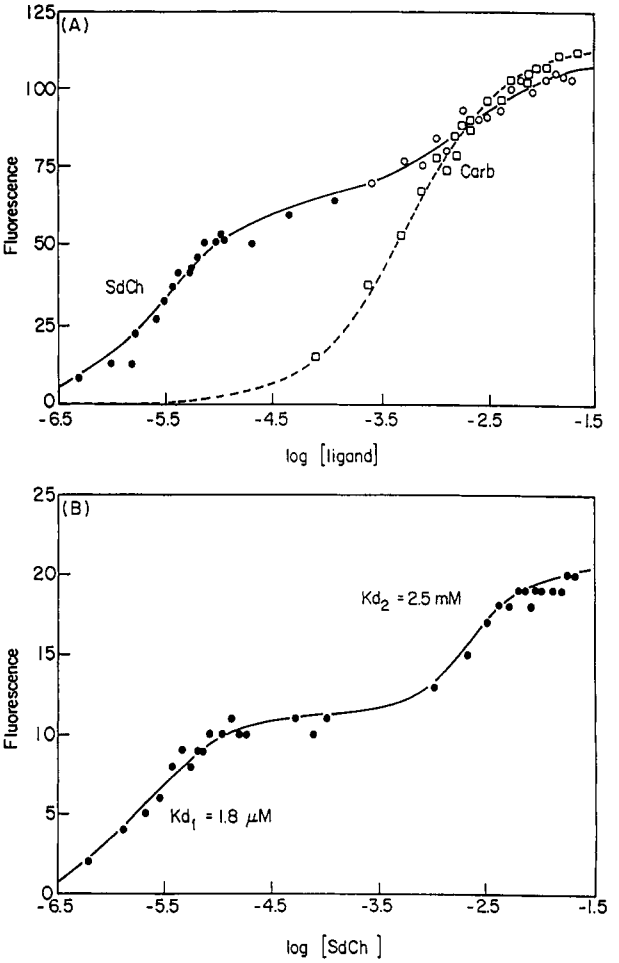


FIGURE 4: Heterogeneity of SdCh binding to NBD-labeled nAcChR. (A) NBD-labeled nAcChR was first titrated by SdCh (●) to give a final concentration of 100 μM, after which the titration was continued using Carb (○). The solid line is calculated from the best fit parameters obtained from fitting by a biphasic model giving a K_{d1} of 2.9 μM (SdCh) and K_{d2} of 1.3 mM (Carb). In the absence of SdCh, titration by Carb (□) gave a K_d of 0.8 mM. (B) Effect of SdCh concentration on the fluorescence of NBD-labeled nAcChR. The solid line is best fit by two independent sites having K_d values of 1.8 μM and 2.5 mM.

fluorescence, and the magnitudes of the ligand-induced fluorescence enhancements were similar to preparations labeled with one of the two probes alone. This is consistent with the results described above that demonstrate that the two fluorophores react at different sites. There is also little spectral overlap between the two probes. IAS fluorescence may be excited directly at 340 nm or indirectly at 290 nm via energy transfer from the protein, resulting in fluorescence emission at about 430 nm whereas the NBD fluorophore absorbs at 480–490 nm and fluoresces at about 535 nm.

Addition of Carb to the doubly labeled preparation shows clearly that the enhancement of IAS fluorescence takes place at low concentration and is saturable in the micromolar range (Figure 5). On the contrary, micromolar concentrations of Carb have no effect on NBD fluorescence, and the fluorescence enhancement of this probe occurs only in the millimolar range (Figure 5). The two probes therefore monitor equilibrium binding to agonist binding sites that have very different affinities.

Equilibrium Binding of [^{3}H]Carbamylcholine to NBD/IAS-Labeled nAcChR. Labeling of nAcChR by IAS, IANBD, or both did not alter either the dissociation constant or the number of high-affinity binding sites for [^{3}H]Carb, as shown for the doubly labeled preparation in Figure 6.

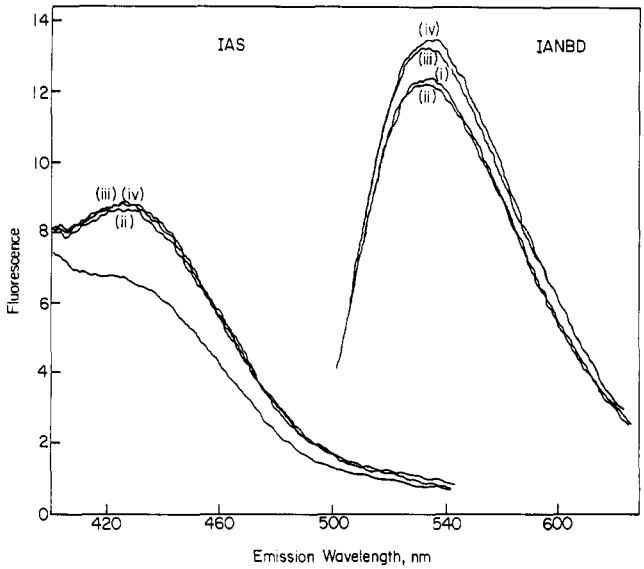


FIGURE 5: Effect of Carb concentration on the fluorescence spectra of double labeled nAcChR (1.4 μM in α-BuTx sites) monitoring either IAS fluorescence (λ_{ex} = 290 nm) or NBD fluorescence (λ_{ex} = 482 nm). Fluorescence spectra of nAcChR alone (i) or in the presence of 25 μM Carb (ii), 2.52 mM Carb (iii), or 10 mM Carb (iv).

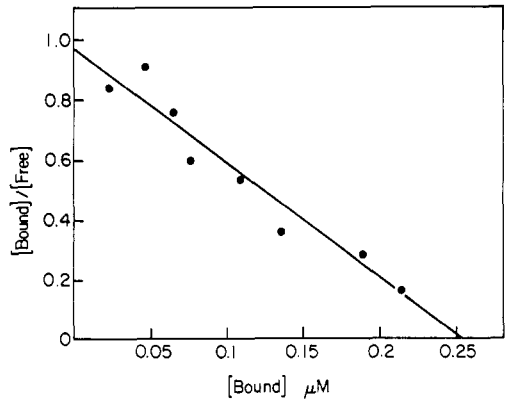


FIGURE 6: Scatchard plot of [^{3}H]Carb binding to NBD/IAS-labeled nAcChR (0.25 μM in α-BuTx sites). The solid line is the linear least-squares fit, giving R_0 = 0.25 μM and K_d = 0.26 μM.

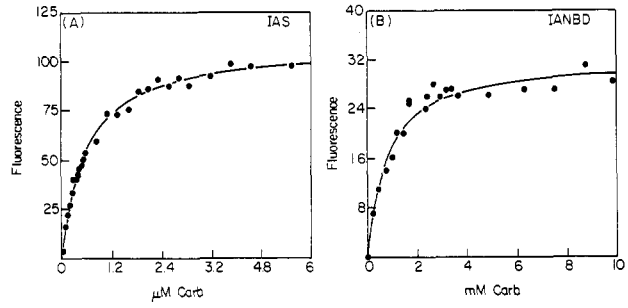


FIGURE 7: Fluorescence titrations of double-labeled nAcChR (0.5 μM in α-BuTx sites). (A) IAS fluorescence (λ_{ex} = 290 nm, λ_{em} = 340 nm). Data were fit by a model with correction for ligand depletion as described in the Materials and Methods, giving K_d = 0.21 μM and R_0 = 0.48 μM. (B) NBD fluorescence (λ_{ex} = 482 nm; λ_{em} = 530 nm). Data were fit by a simple binding model, giving K_d = 0.81 nM. Data were corrected from the results of parallel control titrations of NBD-labeled membranes that had been preincubated with excess α-BuTx.

Equilibrium fluorescence titrations in which the fluorescence of both probes was monitored in parallel show that the K_d estimated from the IAS fluorescence enhancement induced by Carb was 0.2 μM (Figure 7), in good agreement with estimates of radiolabeled Carb binding. The K_d value obtained

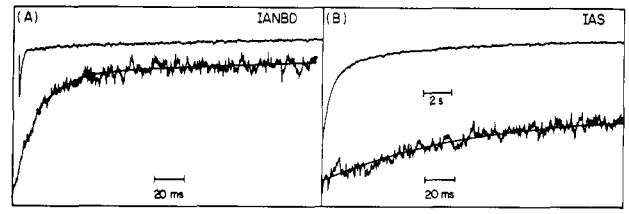


FIGURE 8: Kinetics of Carb (1 mM) binding to double-labeled nAcChR. (A) Changes in NBD fluorescence. The lower trace was fit by single exponential, giving k_{app} = 66 s^{-1}. The upper trace was recorded on a 100 times longer time scale and is offset slightly upward for clarity. (B) Changes in IAS fluorescence. Traces were recorded over the same time courses as in (A), showing the differences in the rate constants. The reaction curve may be fit by two exponential processes having rates of 6.1 s^{-1} and 0.23 s^{-1}.

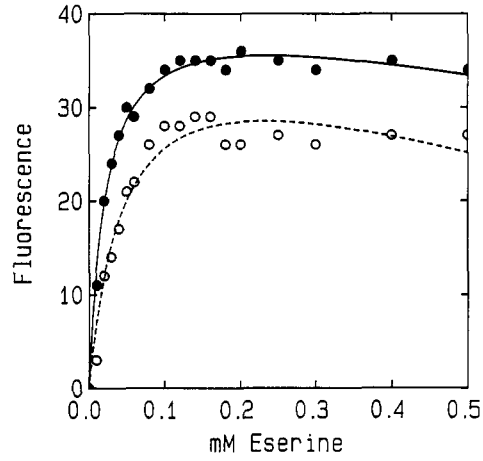


FIGURE 9: Titration of NBD-labeled nAcChR by eserine in the absence (○) or presence (●) of excess α-BuTx. Membranes were titrated with eserine from a concentrated stock solution in ethanol. Data were corrected for nonspecific effects by parallel titrations with ethanol alone. Solid lines are calculated using the best fit parameters from fitting by the equation:

$$Fl = F_0L/(K_d + L) + N_{sp}L$$

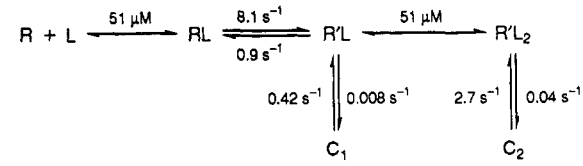
The term $N_{sp}L$ was included to correct for the slight decrease in fluorescence that is routinely observed at high eserine concentrations and is likely to be due to eserine absorbance; K_d values were 23 μM and 48 μM in the absence and presence of α-BuTx, respectively.

from the NBD fluorescence change was 0.81 mM, in agreement with previous results from singly labeled preparations (Dunn & Raftery, 1982a,b).

Kinetics of Agonist Binding to NBD/IAS-Labeled nAcChR. The kinetics of the fluorescence changes occurring on agonist binding to IAS/IANBD-labeled nAcChR have been investigated in stopped-flow fluorescence experiments. Over a range of Carb concentration (0.1–10 mM) the NBD fluorescence enhancement occurred in a single fast phase as shown in Figure 8. This is consistent with previous results in which we have shown that the NBD fluorescence reflects a fast conformational change of the receptor–agonist complex. Using the same preparation but monitoring IAS rather than NBD fluorescence, the enhancement was multiphasic and the rate constants of each phase were much slower than those observed for NBD. At no agonist concentration was there any agreement between the rates obtained from the fluorescence of the two probes.

Effects of Eserine on the Fluorescence of NBD-Labeled nAcChR. Since, with the exception of α-BuTx (see below), the sites reported by changes in NBD fluorescence appear to be specific for receptor ligands that cause channel activation, it was of interest to investigate the effects of eserine, an unconventional nAcChR agonist, on the fluorescence of NBD-labeled receptor. As shown in Figure 9, eserine caused a

Scheme I



saturable enhancement of NBD fluorescence which was not blocked by α-BuTx. The K_d obtained from these and similar equilibrium experiments lay in the range of 20–60 μM, in agreement with the concentrations of eserine that have been reported to induce ion flux (Okonjo et al., 1991).

DISCUSSION

Double labeling of nAcChR preparations by two sulfhydryl alkylating probes, IAS and IANBD, has shown that the two fluorophores react at different locations and reflect agonist binding to distinct binding sites that have very different affinities.

The specific enhancement occurring upon agonist binding to IAS-labeled nAcChR is due to labeling of the α subunit cysteine residues in reduced nAcChR that are also labeled by bromoacetylcholine. Specific labeling was abolished by protection of this disulfide from reduction by inclusion of low concentrations of Carb (10 μM) during the reduction or by prelabeling of the nAcChR by the affinity reagent [^{3}H]-bromoacetylcholine. Quantitation of the extent of IAS labeling by the reduction in the number of residual sites for [^{3}H]BrAcCh has shown that approximately 50% of these sites are routinely labeled. However, covalent attachment of IAS in this position does not perturb the equilibrium binding of radiolabeled agonists, and the fluorescence changes accompanying ligand binding appear to accurately reflect the receptor population as a whole.

Labeling of nAcChR by IANBD also results in significant incorporation of the fluorophore. We have previously reported that, in a typical membrane preparation, approximately three IANBD molecules were incorporated into each nAcChR, and these were associated with the α, β, and γ subunits (Conti-Tronconi et al., 1982). Although the labeled sites that give rise to the NBD fluorescence changes occurring on agonist binding have not been identified and cannot presently be quantitated, it is clear tht IANBD does not react with the cysteines generated by reduction of the disulfide bond near the α subunit binding sites.

When nAcChR preparations were labeled by both IAS and IANBD, fluorescence titration data clearly demonstrate that the two probes are monitoring agonist binding to two classes of sites having different affinities (K_ds of 0.2 μM and 0.8 mM for Carb). The kinetics of the fluorescence changes were also quite different for the two fluorophores. At high Carb concentrations, the NBD signal change was monophasic and rapid (K_{app} = 66 s^{-1} at 1 mM). The IAS fluorescence enhancement was more complex and could be resolved into several phases, all of which had rates appreciably less than that observed with NBD. We have previously reported a detailed analysis of the concentration dependence of Carb binding to IAS-labeled nAcChR and proposed the model shown in Scheme I (Dunn et al., 1980). According to this model, the pathway to C_1 corresponds to the high-affinity binding measured in fluorescence titrations or in the equilibrium binding of radiolabeled agonists. All of the observed conformational transitions are too slow to be rate-limiting steps in channel activation, and also the extrapolated dissociation constants for the resting state are much lower than concentrations of agonist that are required to induce ion flux. Desensitization has been shown in electrophysiological experiments to be characterized by several rate constants similar to those shown above (Sakmann et al., 1980; Anwyl & Narahashi, 1980; Feltz & Trautmann, 1980, 1982), and it is therefore likely that IAS fluorescence reflects similar desensitization processes. Conformational changes associated with binding to these sites do not, therefore, display the characteristics of being directly involved in channel opening.

In the same preparations, agonist-induced changes in fluorescence of bound NBD show clearly that the nAcChR has a distinct class of low-affinity binding sites that are present at equilibrium, i.e., in desensitized nAcChR. The close agreement between dissociation constants obtained in NBD fluorescence titrations and from ion flux measurements (Dunn & Raftery, 1982a,b), in addition to the rapidity of the agonist-induced conformational changes, is compelling evidence that these sites are functionally important in channel activation. We have previously proposed a model (Raftery et al., 1983) according to which channel activation and desensitization are parallel processes mediated by agonist binding to distinct binding sites. Occupancy of the low-affinity sites results in a rapid conformational transition which leads to channel activation. Although, if the agonist concentration remains high, these sites will remain occupied, parallel processes mediated by agonist binding to other sites (R in Scheme I above) result in the slower conformational changes that desensitize the receptor.

Although there is strong evidence for the existence of low-affinity agonist sites, proving a functional role is much more difficult. In this respect, it is intriguing that eserine, an unconventional agonist, also gives rise to an NBD fluorescence change. In electrophysiological experiments, eserine has been shown to be an activator of nAcChR at the neuromuscular junction (Pascuzzo et al., 1984; Albuquerque et al., 1989), and more recently, it has been reported to stimulate ion flux in nAcChR-enriched membrane vesicles from *Torpedo* electric organ (Okonjo et al., 1991). An unusual property of the eserine-induced flux response in *Torpedo* nAcChR is that it was not blocked by α-BuTx. In the present study, it has been shown that eserine induces an NBD signal change in labeled nAcChR with a similar dose dependence to the reported flux response (Okonjo et al., 1991) and, like the flux response, this was not blocked by α-BuTx. In the case of classical agonists, both flux responses and changes in the fluorescence of NBD-labeled nAcChR are blocked by α-BuTx (Dunn & Raftery, 1982a,b). Taken together, these results suggest the existence of a binding site(s) for eserine that may be involved in channel activation but is distinct from the high-affinity sites that bind agonists, antagonists, and α-BuTx. The binding site(s) for eserine that leads to channel activation also appears to be distinct from the low-affinity sites for classical cholinergic agonists since it has been reported that the eserine-induced flux response is not blocked by α-BuTx. Nevertheless, the binding of both classical agonists and eserine elicit conformational changes detected by NBD fluorescence changes with concentration dependencies very similar to those that elicit channel activation.

At the neuromuscular junction, the concentration of acetylcholine soon after release is likely to be high (Land et al., 1980, 1984) and sufficient to occupy low-affinity sites, leading to a rapid conformational change and channel opening. At the same time, all available sites, including those associated with the α subunits, will be occupied since the rates of the bimolecular association reactions are likely to be close to diffusion controlled. Eventually, as a result of slow confor-

mational changes *in vitro*, the *Torpedo* nAcChR becomes desensitized and it assumes a conformation with high affinity for agonists. However, the distribution of resting state, activated state, and close/desensitized state 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 to the short time of occupancy of the sites as a consequence of rapid diffusion of acetylcholine out of the synaptic cleft and its destruction 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.

In conclusion, the *Torpedo* nAcChR appears to carry multiple binding sites for cholinergic agonists. The recent demonstrations that individual subunits of the $GABA_A$ (Blair et al., 1988), glycine (Betz, 1991), and $5HT_3$ (Maricq et al., 1991) receptors are capable of forming homo-oligomeric agonist-gated ion channels indicate that these receptors must also carry multiple agonist binding sites. This suggests that further characterization of agonist binding of nAcChR may shed light on the mechanisms of action of other members of this ligand-gated ion channel family.

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