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INDEPENDENT SITES OF LOW AND HIGH AFFINITY FOR AGONISTS ON TORPEDO CALIFORNICA ACETYLCHOLINE RECEPTOR

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SUMMARY: It is demonstrated that two classes of binding site for acetylcholine are present on Torpedo californica acetylcholine receptor. One class is the well documented site on each of the two subunits of 40,000 daltons, which can be covalently modified by bromoacetylcholine. Both in the absence and in the presence of bromoacetylcholine another binding site is shown to exist by virtue of acetylcholine dependent fluorescence changes in the receptor covalently modified by 4-[N-(iodoacetoxy)ethyl-N-methyl]-amino-7-Nitrobenz-2-oxa-1,3 diazole (IANBD). This site has a low affinity for acetylcholine ($K_d \sim 80$ μM) that corresponds closely with the known concentration dependence of acetylcholine mediated activation of this receptor and we conclude that it may represent a site of association that participates in channel opening in this system.

INTRODUCTION: The function of the postsynaptic nicotinic acetylcholine receptor (AcChR) in muscle and electroplax is to mediate, in response to the binding of agonists, a rapid ion flux through a cation selective channel which is part of the AcChR molecule (1). A major goal in elucidation of the mechanism of the AcChR has been to define the ligand binding events leading to activation of the cation channel. Quantitative in vitro studies of the ligand binding and cation

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ABBREVIATIONS

AcChR	acetylcholine receptor	BrAcCh	bromoacetylcholine
IANBD	4-[N-(iodoacetoxy)ethyl-N-Methyl]-amino-7-Nitrobenz-2-oxa-1,3 diazole		
MBTA	maleinidobenzyltrimethylammonium iodide	AcCh	acetylcholine
α-BuTx	α-bungarotoxin	DEAE	diethylaminoethyl
DTT	dithiothreitol		
SDS	sodium dodecylsulfate	EDTA	ethylenediamine tetraacetic acid
NBD	Nitrobenz-2-oxa-1,3 diazole		

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gating properties of purified, membrane bound Torpedo AcChR are possible due to the availability of vesicle preparations containing only the AcChR protein (2-5) and it has recently been shown that such preparations are fully functional (6) allowing the conclusion that the AcChR alone, composed of four homologous polypeptides (8, 9) constitutes the complete physiological AcChR. Upon exposure to cholinergic agonists, Torpedo AcChR becomes rapidly activated in vitro to cause a rapid ion flux (6, 7) and in addition undergoes a slow conformational change (timescale of seconds) to a state of higher affinity for these ligands (10-14), a transition which has been correlated with the phenomenon of desensitization (15) in vivo.

Attempts have been made to correlate the results of direct ligand binding studies in vitro with the agonist concentration dependence of conductance changes measured by electrophysiological techniques in vivo and by rapid kinetic measurements in vitro. The lack of agreement between measured dissociation constants for binding to the resting or high affinity desensitized states of the AcChR and those characterizing the dose dependence of conductance changes has been a major problem (see 1). Complex ligand binding mechanisms which involve sequential transitions of affinity of one or two binding sites have been proposed (7, 16).

A binding site for agonists and antagonists has been localized on the subunit of lowest M_r (approx. 40,000 daltons) in AcChR from different species, since, after reduction of a reactive disulphide bond near this site, it can be alkylated by either the agonist bromoacetylcholine (BrAcCh) (17-20) or other affinity reagents such as maleimidobenzyltrimethylammonium iodide (MBTA) (21). It has been assumed generally that agonist binding to these sites leads to both channel activation and to desensitization.

An alternative possibility is that the AcChR molecule has multiple binding sites for agonists, each of which may have different functional properties. This is not unlikely since the AcChR molecule is formed, in all the species so far examined, by four structurally related subunits (of M_r 40, 50, 60 and 65K in Torpedo californica AcChR; 41, 50, 55 and 64K in Electrophorus electricus

AcChR and 42, 49, 54 and 58K in fetal calf muscle AcChR) in a stoichiometry of 2:1:1:1 (8, 9, 22). The presence of five homologous domains on the surface of the AcChR molecule leads to the idea of possible multiple binding sites for cholinergic ligands.

In the present communication we directly show that the AcChR contains a binding site(s) distinct from those previously characterized by labeling of the 40K subunits by BrAcCh or MBTA. Furthermore, the affinity of this site for acetylcholine (AcCh) corresponds closely with the known affinity of this agonist for a site(s) causing depolarization.

METHODS: Postsynaptic membrane fragments from T. californica electroplax containing the AcChR as the only major protein component (Figure 1, scan 2) were purified by sucrose gradient centrifugation and subsequent alkali extraction of peripheral proteins (4, 5). The concentration of α-Bungarotoxin (α-BuTx) binding sites was measured by the DEAE disc assay (23). For labeling with IANBD these membranes [10^{-5} M in α-BuTx binding sites] were reduced with $5x10^{-5}$ M DTT in 10 mM Hepes, 35 mM $NaNO_3$ pH 7.4 and alkylated with approximately $3x10^{-5}$ M IANBD (24). The labeled membranes were solubilized in SDS and submitted to SDS-polyacrylamide gel electrophoresis (25). The labeling pattern was investigated by scanning unstained gel strips at 480 nm for IANBD absorbance. These scans were compared with the densitometric scans at 550 nm of the same preparation after Coomassie blue staining for proteins.

For covalent reaction of NBD-labeled AcChR with ^{3}H-BrAcCh (19) membrane fragments (~1 μM in α-BuTx sites) were first reduced with 0.3 mM DTT under argon in 15 mM Tris-HCl, 150 mM NaCl, 4.5 mM NaN_3, 1.5 mM EDTA, pH 8 for 45 minutes at room temperature (20). Following removal of DTT by centrifugation membrane fragments were reacted with 40 μM ^{3}H-BrAcCh and centrifuged again to remove free reagent. The extent of labelling was estimated by DE81 disc assay (23) using as control a parallel sample which had been incubated with excess α-BuTx prior to addition of ^{3}H-BrAcCh.

RESULTS AND DISCUSSION: Evidence for agonist binding sites of low affinity in T. californica AcChR has recently been obtained by studying the effects of cholinergic ligands on the fluorescence of a probe (IANBD) covalently attached to the receptor protein (23). Titrations of AcChR rich membranes labeled with this probe revealed low affinity sites specific for agonists (K_d for carbamylcholine ~1 mM). Since this low affinity binding site is still present in preparations desensitized by incubation with low concentrations of carbamylcholine it is possible to study its properties under equilibrium conditions.

We have therefore conducted studies designed to determine the relationship, if any, between the well characterized binding sites for BrAcCh on the 40K dalton subunits and the low affinity binding site(s) revealed by IANBD.

Figure 1 shows the labeling of AcChR subunits by IANBD and indicates that the 40 and 50K dalton polypeptides were the major AcChR subunits labeled by the reagent with lesser but significant labeling of the 60K subunit. A non-receptor protein of M_r approximately 100K, present in small amounts, was also labeled by the alkylating fluorophore. This NBD labeled AcChR preparation was next labeled by [^{3}H]-BrAcCh. Maximal labeling was achieved under the conditions used, i.e. the number of sites labeled by BrAcCh were equivalent to the number of binding sites for α-BuTx (see also 20). Prior labeling of AcChR by IANBD (using $5x10^{-5}$ M DTT) had no effect on the extent of subsequent maximal BrAcCh labeling (using $3x10^{-5}$ M DTT). Thus the same site was not labeled by both reagents and therefore IANBD reacts at a location removed from the BrAcCh binding sites.

The effect of acetylcholine (AcCh) on the NBD fluorescence of control, DTT-reduced (0.3 mM) and BrAcCh labeled AcChR-rich membranes which had all been prelabeled with IANBD was investigated and the results are shown in Figure 2. In each case an enhancement of fluorescence was observed and the apparent dissociation constant for AcCh (control, Kd = 0.12 ± 0.01 mM; DTT reduced, Kd = 0.21 + 0.01 mM; DTT reduced, BrAcCh reacted, Kd = 0.055 ± 0.001 mM) was not affected either by DTT reduction or by reduction followed by alkylation with BrAcCh. Maximal labeling with BrAcCh therefore does not perturb AcCh binding to the low affinity site revealed by the fluorescent probe.

This demonstrates the existence of two classes of AcCh binding sites, one of which has high affinity and is labelled by BrAcCh on two of the five AcChR subunits (8, 9) and another which has much lower affinity and is revealed by changes in IANBD fluorescence. The fact that subunits other than those of 40K were labeled by IANBD as shown here opens the possibility that the low affinity AcCh binding site may reside partially or completely on a different subunit(s) i.e. of 50, 60 or 65K. The equilibrium and kinetic properties of agonist binding to the low affinity site (24) closely correlate with those observed for the ligand dependence of channel activation for T. californica AcChR (6, 7). An attractive possibility is that binding of AcCh to these two classes of site mediates different functions, namely, activation and desensitization, the latter

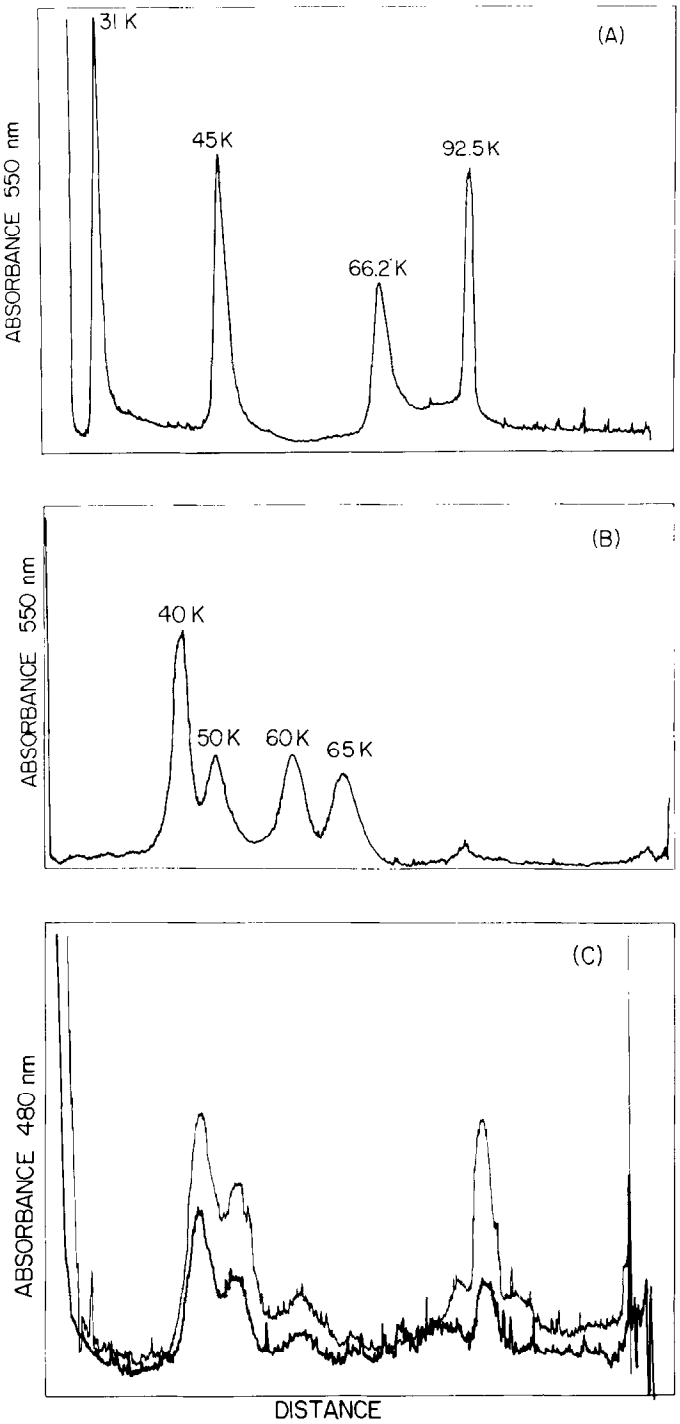

Figure 1: Polyacrylamide gel electrophoresis in sodium dodecyl sulfate: (A) standards; (B) T. californica AcChR subunits stained with Coomassie Brilliant Blue: (C) same as in (B) prior to staining. Absorbance of covalently attached NBD, demonstrating that the subunits of 40, 50 and 60K contained NBD residues. Upper trace- 2 mg NBD- AcChR loaded and lower trace 1 mg AcChR loaded.

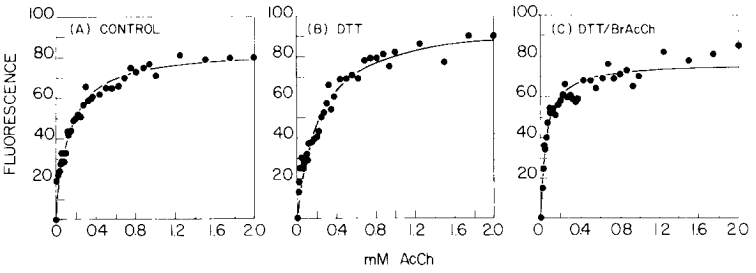

Figure 2: Titration of NBD labeled AcChR with acetylcholine: (A) control NBD-AcChR; (B) DTT (0.3 mM) reduced NBD-AcChR; (C) DTT reduced and bromo-acetylcholine alkylated NBD-AcChR. Excitation and emission wavelengths were 482 and 540 nm respectively. Data were corrected for non-specific effects by parallel titration of the sample reacted with α-bungarotoxin. Titration data were fit using a non-linear regression program (26). Quantitation yielded values of Fo (fluorescence enhancement in arbitrary units) and Kd for the three preparations as follows:
(A) Fo = 83.2 ± 0.1, Kd = 0.12 ± 0.01 mM;
(B) Fo = 97.3 ± 0.6, Kd = 0.21 ± 0.01 mM;
(C) Fo = 76.3 ± 0.3, Kd = 0.055 ± 0.001 mM.

process possibly involving the site labelled by BrAcCh. A model that suggests independent parallel pathways for activation and desensitization adequately explains the data obtained for T. californica AcChR and the fact that AcChRs from all the animal species and tissues studied so far have the same molecular structure formed by homologous subunits (8, 9) makes it likely that multiple binding sites exist on AcChRs from many or all creatures. However, species variation, despite the high degree of structural homology, might be expected both in binding parameters and specific function of each site. Such issues should be clarified for each species studied in the future.

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