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Epibatidine binds to four sites on the *Torpedo* nicotinic acetylcholine receptor

Hideki Kawai [a,1], Susan M.J. Dunn [b,*], Michael A. Raftery [c,2]

[a] *Department of Pharmacology, University of Minnesota, Minneapolis, MN 55455, USA*
[b] *Department of Pharmacology, University of Alberta, Edmonton, Alta., Canada T6G 2H7*
[c] *Department of Biochemistry, Molecular Biology and Biophysics, University of Minnesota, St. Paul, MN 55108, USA*

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Abstract

The nicotinic acetylcholine receptor (nAChR) from *Torpedo* electric organ is a pentamer of homologous subunits. This receptor is generally thought to carry two high affinity sites for agonists under equilibrium conditions. Here we demonstrate directly that each *Torpedo* nAChR carries at least *four* binding sites for the potent neuronal nAChR agonist, epibatidine, i.e., twice as many sites as for α-bungarotoxin. Using radiolabeled ligand binding techniques, we show that the binding of [^{3}H]-(±)-epibatidine is heterogeneous and is characterized by two classes of binding sites with equilibrium dissociation constants of about 15 nM and 1 μM. These classes of sites exist in approximately equal numbers and all [^{3}H]-(±)-epibatidine binding is competitively displaced by acetylcholine, suberyldicholine and *d*-tubocurarine. These results provide further evidence for the complexity of agonist binding to the nAChR and underscore the difficulties in determining simple relationships between site occupancy and functional responses.

Keywords: Nicotinic; Acetylcholine; Epibatidine; α-Bungarotoxin; Suberyldicholine; Receptor binding sites; Receptor function; Radiolabelled ligand binding; *Torpedo californica*

Detailed characterization of the *Torpedo* nAChR, the prototype of the 'cys-loop' family of ligand-gated ion channels see [1], has been facilitated by its abundance in the electric organ and its defined subunit structure ($\alpha_2\beta\gamma\delta$). The binding of agonists and competitive antagonists to the nAChR has been extensively scrutinized. It is now generally agreed that, under equilibrium conditions, each receptor carries two high affinity binding sites and that these sites are formed by discrete 'loops' of amino acids that lie at the α−γ and α−δ subunit interfaces [2–4]. The location of these sites has been corroborated by elucidation of the crystal structures of related acetylcholine binding proteins (AChBPs) e.g. [5–7]. There is, however, a body of information to indicate that the binding sites may be more complex than is readily apparent from static structures. In early studies, for example, the binding of bisquaternary amines to solubilized receptors suggested multiple subsites for ligand interaction [8]. Additionally, the ability of various monoclonal antibodies to differentially block the binding of different classes of cholinergic ligands suggested the presence of multiple, possibly three, distinct subsites within each high affinity binding domain [9]. Later, studies of the kinetics of association and dissociation of radiolabeled ACh and the bisquaternary agonist, suberyldicholine (SbCh), again suggested that each high affinity site may be made up of two subsites [10,11].

In addition to the complexity of subsites within the high affinity site, we have previously presented evidence for distinct agonist binding sites on the nAChR. We iden-

* Corresponding author. Fax: +1 780 492 4325.
E-mail address: susan.dunn@ualberta.ca (S.M.J. Dunn).
[1] Present address: Department of Neurobiology and Behavior, University of California, Irvine, CA 92697, USA.
[2] In memory of Michael A. Raftery.

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tified low affinity agonist-specific sites, which displayed properties that were consistent with the involvement of these sites in channel activation [12–14]. Supporting evidence for multiple binding sites has come from studies of α-dendrotoxin binding [15], photoaffinity labeling [16], and from flux studies of pre-formed receptor–ligand complexes [17]. In this report, we have characterized the binding of an agonist, epibatidine [18], to the *Torpedo* nAChR and directly demonstrate the presence of four agonist binding sites under equilibrium conditions.

Materials and methods

Unlabeled ligands. (±)-Epibatidine HCl was obtained from Tocris (Ballwin, MO), Sigma–Aldrich Canada Ltd. (Oakville, Ontario) or RBI (Natick, MA). (+)-Epibatidine L-tartrate, carbamylcholine chloride, acetylcholine chloride, diethyl-*p*-nitrophenylphosphate (DNPP), chlorpromazine hydrochloride, and *d*-tubocurarine (*d*-TC) were also from Sigma. Suberyldicholine dichloride was from Aldrich and methyltriphenylphosphoium bromide (MTPP-Br) from Pfaltz and Bauer, Inc. β-Erythroidine was a generous gift from Dr. Virgil Boekelheide (University of Oregon, OR).

Radiolabeled ligands. [^{125}I]α-BTx was from DuPont Canada or DuPont USA and was calibrated as previously described [19]. [^{3}H]-ACh (specific activity = 46.9 mCi/mmol) was from DuPont-NEN Canada and [^{3}H]-SbCh (74 mCi/mmol) was synthesized and its specific activity determined as described [10]. [^{3}H]-(±)-epibatidine (two batches of 33.8 and 66.6 Ci/mmol) was obtained from New England Nuclear Life Sciences (Boston, MA) and was isotopically diluted to make stock solutions of 1 mM with a specific activity of 48–50 mCi/mmol. The concentration of labeled (±)-epibatidine was determined by its absorbance at 268 nm ($E_{mM} = 0.384$) and at 217 nm ($E_{mM} = 0.96$). The results presented here rely on the accurate calibration of the [^{3}H]-(±)-epibatidine stock solutions. Extinction coefficients were, therefore, determined independently in our two laboratories using unlabeled (±)-epibatidine of different batches from three different sources. The purity of tritiated ligands was examined by paper (Whatman #1) chromatography in 70% propanol. Staining for amines using the Dragendorff reagent [20] and counting of strips for radioactivity identified one location, demonstrating isotopic purity.

Preparation of nAChR-enriched membrane fragments. Membranes were prepared from frozen electric organ of *Torpedo californica* (Biomarine, San Pedro, CA or Aquatic Consultants, San Pedro, CA) as described previously [21] and stored frozen at −85 °C. Prior to use, the membranes were alkali-extracted to remove peripheral proteins [22,23] and were finally resuspended in Ca^{2+} free *Torpedo* Ringers (20 mM Hepes, 250 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 0.02% NaN_3, pH 7.4). Protein concentrations were measured by the BioRad assay and the concentration of [^{125}I]α-BTx sites was measured by DEAE-disc assay [24]. Specific activities of the *Torpedo* receptor preparations were in the range of 1–3 nmol of [^{125}I]α-BTx sites per milligram of protein.

Equilibrium radiolabeled ligand binding measurements. Prior to binding experiments, acetylcholinesterase activity was inhibited by incubating concentrated membrane preparations (approximately 10 μM in α-BTx binding sites) with 0.5% volume of a stock solution of 0.3 M DNPP in isopropanol for 3 min at room temperature [10]. Following treatment, the membranes were diluted approximately 50-fold in *Torpedo* Ringers containing 4 mM $CaCl_2$ and stored on ice until use. The equilibrium binding of radiolabeled ligands was routinely measured by centrifugation assays [25]. In saturation binding experiments, the radioligand was incubated with DNPP-treated *Torpedo* membranes for 30 min at room temperature followed by centrifugation for 20 min in a microcentrifuge at 12000*g* and 4 °C. Total and free radioligand concentrations were estimated from counting duplicate aliquots (75 μl each) of the original sample and of the supernatant after centrifugation. Non-specific binding was estimated from the results of parallel samples in which a large excess of unlabeled ligand was included in the incubation mixture. The displacement of radioligands by unlabeled ligands was measured in competition experiments using a similar centrifugation assay.

In experiments in which higher concentrations of receptor were used, binding was measured by equilibrium dialysis using microdialysis chambers (Chemical Rubber Company) and 50 K molecular weight cutoff dialysis tubing (SpectraPor). The DNPP-treated membranes (0.4 ml per sample) were dialyzed against 0.4 ml radiolabeled ligand for 6 h with rocking at 4 °C [10]. Total and free radioligand concentrations were estimated by taking duplicate 50 μl samples from each compartment and counting for radioactivity. Non-specific binding was measured in the presence of a large excess of unlabeled ligand.

Data analysis. All data were analyzed using Prism 3 software (GraphPad Inc., San Diego). Saturation binding data were analyzed using either a one-site or two-site hyperbolic function:

$$[B] = B_{max1}[F]/(K_{d1} + [F]) + B_{max2}[F]/(K_{d2} + [F]) + NSP^*[F]$$

where [B] is the concentration of bound radioligand, [F] is the concentration of free radioligand, B_{max1} and B_{max2} are the concentrations of the two classes of binding sites, K_{d1} and K_{d2} are their corresponding equilibrium dissociation experiments and NSP is non-specific binding. Competition binding data were fit by either a competition model with variable Hill coefficient (n_H):

$$[B] = [B_{min}] + [B_{max} - B_{min}]/\left[1 + \left(10^{\log[X]}/10^{\log IC_{50}}\right)^{n_H}\right]$$

or a two component model:

$$[B] = [B_{min}] + \{[B_{max}] - [B_{min}]\} * \left\{F_H \Big/ \left(1 + 10^{\log[X]}/10^{\log IC_{50(1)}}\right) + (100 - F_H) \Big/ \left(1 + 10^{\log[X]}/10^{\log IC_{50(2)}}\right)\right\}$$

where [B] is bound radioligand, B_{min} and B_{max} are the minimum and maximum amount of bound radioligand, [X] is the total concentration of competing ligand, F_H is the fraction of high affinity sites and $IC_{50(1)}$ and $IC_{50(2)}$ are the IC_{50} values for the high and low affinity components, respectively.

Results

Epibatidine binds to four sites per receptor molecule

Initial experiments to characterize the equilibrium binding of [^{3}H]-(±)-epibatidine to the *Torpedo* nAChR were carried out using centrifugation assays and relatively low receptor concentrations (0.1–0.5 μM in [^{125}I]α-BTx sites). The binding of [^{3}H]-(±)-epibatidine was obviously heterogeneous and non-linear regression curve fitting indicated the presence of two populations of sites that existed in approximately equal proportions (Fig. 1A). A Scatchard plot of these data (Fig. 1B) more clearly illustrates this binding site heterogeneity. Averaged data gave affinity estimates of 15.2 ± 3.1 nM and 0.42 ± 0.05 μM (mean ± SEM, $n = 6$) for the two classes of sites. The most important finding is that the total number of binding sites exceeds those for α-BTx by a factor of 2. Since α-BTx is known to bind to two sites per receptor, these results indicate that there are four measurable sites for [^{3}H]-(±)-epibatidine on each nAChR. In order to eliminate complications that could possibly have arisen from inaccurate determinations of the toxin specific activity, most experiments included parallel studies of [^{3}H]-ACh and [^{3}H]-SbCh binding and the results demonstrated that, as expected, these ligands bind to an equivalent number of sites as [^{125}I]α-BTx. Thus the