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Barbiturates Bind to an Allosteric Regulatory Site on Nicotinic Acetylcholine Receptor-Rich Membranes

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SUMMARY

The ability of barbiturates to bind to acetylcholine receptor-rich membranes purified from the electroplaques of *Torpedo nobiliana* was examined by centrifugation assay. [^{14}C]Amobarbital both partitioned into the membrane and bound displaceably to a site with an equilibrium dissociation constant of 12 μM. This low affinity made the stoichiometry difficult to obtain despite the high specific activity of acetylcholine receptors in this membrane preparation. However, the data are not inconsistent with a stoichiometry of one barbiturate-binding site per acetylcholine-binding site. Displaceable [^{14}C]amobarbital binding was completely inhibited by barbiturates (IC_{50}: amobarbital, 28 μM; secobarbital, 110 μM; pentobarbital, 400 μM; phenobarbital, 690 μM; butabarbital, 690 μM; and barbital, 5.1 mM. α-Bungarotoxin had no effect, but cholinergic ligands that convert the acetylcholine receptor to the desensitized state (acetylcholine, carbamylcholine, and, to a lesser extent, *d*-tubocararine) partially inhibited displaceable [^{14}C]amobarbital binding. This cholinergic inhibition was prevented by preincubation with α-bungarotoxin, implying an allosteric mediation through the classical cholinergic site. This negative interaction between the cholinergic and the barbiturate sites was mutual with barbiturates partially decreasing equilibrium [^{3}H]acetylcholine binding in a saturable fashion with relative affinities that parallel those for inhibiting [^{14}C]amobarbital binding (IC_{50}). These data establish a mutual negative heterotropic interaction between barbiturate-binding sites and cholinergic binding sites on the nicotinic acetylcholine receptor from *Torpedo*.

The neurochemical basis for the clinical actions of barbiturates remains uncertain despite their extensive use in medicine. Barbiturates are capable of modulating neuronal transmission by both enhancing postsynaptic inhibitory responses and inhibiting postsynaptic excitatory responses (for reviews see Refs. 1 and 2). Their anesthetic potencies are proportional to the lipid solubility of the unionized species (3), supporting a nonspecific mechanism of action (4). However, specificities have been noted in some barbiturate actions. For example, barbiturates allosterically enhance the binding of GABA agonists and benzodiazepine agonists to the GABA receptor-ionophore complex (5, 6). This enhancement is structurally specific, stereoselective, and inhibited by picrotoxinin (7, 8). Conversely, picrotoxinin binding is itself inhibited by barbiturates (9). Unfortunately, the complexity of the interactions, as well as the low density of neuronal GABA receptors, has made a direct examination of this barbiturate-binding site technically difficult (9).

Similar problems beset the study of barbiturate interactions with central excitatory synapses. Fortunately, there is considerable structural homology between central nicotinic cholinergic receptors and the postsynaptic acetylcholine receptors purified from *Torpedo* electroplaques (10). The high specific activity of the *Torpedo* receptor-rich membranes [1–2 μmol of acetylcholine-binding sites/g of protein in *Torpedo* versus ~70 pmol/g of protein in the central nervous system (11)] permits the examination of binding sites not detectable in the central nervous system. Alcohols and volatile general anesthetics desensitize *Torpedo* acetylcholine receptors by increasing the binding affinities of the cholinergic agonists in a manner proportional to the membrane/buffer partition coefficient of the anesthetic agent (12–14). In contrast, pentobarbital decreases the equilibrium binding of [^{3}H]acetylcholine by approximately 50% in a concentration-dependent, saturable fashion, with a half-effect concentration, EC_{50}, of about 200 μM (15). Pentobarbital also displaces [^{14}C]pentobarbital bound to the acetylcholine receptor in a concentration-dependent, stereoselective fashion with a half-inhibition concentration, IC_{50}, of about 200 μM (15). Although α-BTX and DFP were without effect, both carbamylcholine and *d*-turbocurarine reduced total [^{14}C]pentobarbital binding by 20%. Increasing the pH reduced both displaceable and nondisplaceable binding, consistent with the uncharged barbiturate being the active species. However, characterization of the functional significance of the pentobarbital site was difficult because of the racemic radiolabeled ligand and because the ratio of displaceable to nondisplaceable binding was only about 1.4 (15).

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ABBREVIATIONS: GABA, γ-aminobutyric acid; α-BTX, α-bungarotoxin; DFP, diisopropyfluorophosphate.

This study compares the ability of six clinically used barbiturates to alter cholinergic binding based on their ability to bind specifically to the acetylcholine receptor. The achiral barbiturate, amobarbital, has both the highest efficacy for decreasing acetylcholine binding and the highest affinity for binding to *Torpedo* acetylcholine receptors (16). The ratio of displaceable to nondisplaceable [^{14}C]amobarbital binding is approximately 2.5 (16), which allowed a more detailed characterization of barbiturate-cholinergic interactions.

Experimental Procedures

Materials. [^{3}H]Acetylcholine (0.5–3.2 Ci/mmol) was obtained from New England Nuclear Corp. (Boston, MA) and Amersham-Searle (Arlington Heights, IL). The specific activity was confirmed by isotope dilution experiments as described by Neubig and Cohen (17). [^{14}C] Pentobarbital (52 mCi/mmol) and [^{3}H]*d*-tubocurarine (15.8 Ci/mmol) were purchased from New England Nuclear. [^{14}C]Pentobarbital (21.8 mCi/mmol) was purchased from ICN Pharmaceuticals, (Irvine, CA). [^{14}C]Amobarbital (52 mCi/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO). α-BTX was purchased from Miami Serpentarium (Miami, FL). *Torpedo nobiliana* were purchased from Biofish Associates (Georgetown, MA). All other drugs and materials were obtained from commercial sources.

Preparation of membranes. Acetylcholine receptor-rich membranes were prepared from freshly dissected *T. nobiliana* electroplaques by differential and sucrose density gradient centrifugation, as described previously (18). These membranes contained 1–2 μmol of acetylcholine-binding sites/g of protein. Membrane stocks used within 3 weeks were stored under nitrogen at 4°. Membranes requiring a more prolonged storage were suspended in 0.3 M sucrose, frozen in liquid nitrogen, and stored at −85° until needed. Neither storage condition significantly affected the results.

Equilibrium binding of [^{3}H]acetylcholine. Membranes were first incubated for 30 min with DFP (100 μM) to inactivate acetylcholinesterase. [^{3}H]Acetylcholine binding to pretreated membranes was then determined after a 30-min equilibration period at room temperature in *Torpedo* Ringers (250 mM NaCl, 5 mM KCl, 3 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM Na_2PO_4, pH 7.0), with or without barbiturates. Bound and free ligands were separated either by centrifugation or filtration. In the centrifugation assay, 150-μl samples were placed into microcentrifuge tubes and centrifuged (Beckman Airfuge Ultracentrifuge, 30° rotor, model A-100, 133,000 × *g*, 30 min), and the resultant supernant was carefully aspirated. Three washes, in which 150-μl aliquots of ice-cold *Torpedo* Ringers were added to the centrifuge tube for 10 sec and then carefully aspirated, were performed immediately to remove any remaining supernatant (i.e., free ligand). To solubilize the pellet, 150-μl aliquots of 10% sodium dodecyl sulfate were added to the centrifuge tubes, the tubes were transferred to 7-ml vials containing 6 ml of scintillation cocktail (see below), and vials were exposed to 37° for at least 1 hr. The vials were then vortexed vigorously and counted. In the filtration assay (18), the filters (Whatman GF/F glass fiber filters) were not washed because of the possibility of rapidly reversible binding (19). In this case, total binding was determined as the difference between the total and free concentrations. Binding occurring in the presence of a 10-fold excess of α-BTX was defined as nonspecific and accounted for approximately 4% of total bound [^{3}H]acetylcholine.

Binding of ^{14}C-labeled barbiturates. [^{14}C]Amobarbital or [^{14}C] pentobarbital binding to membranes was determined by centrifugation assay (see above) after a 30-min equilibration interval at room temperature in *Torpedo* Ringers, with or without barbiturates, tritium-labeled, and/or unlabeled cholinergic ligands. When appropriate, the membranes were pretreated with DFP. Final control conditions were determined to produce the maximum displaceable binding; that is, the maximum difference in ^{14}C-barbiturate bound in the presence and absence of excess amobarbital (1 mM). Specific binding is expressed as percentage of displaceable binding.

Data analysis. All samples were counted in 7-ml glass vials containing 6 ml of Liquiscint scintillation cocktail (National Diagonistics, Somerville, NJ) in a Beckman LS 8100 scintillation counter. Efficiency curves were obtained by the external standard method (20) using commercially available [^{14}C] and [^{3}H] standards. The [^{14}C] channel was set to exclude [^{3}H] counts in the double isotope experiments. [^{14}C] spillover into the [^{3}H] window was corrected for by the isotope exclusion method (20) or from matched unlabeled amobarbital samples.

Concentration-response relationships were fitted to the logistic function,

$$E = M\left(\frac{A^P}{A^P + K^P}\right) \quad (1)$$

which is the sigmoid-shaped function with a maximum of M, EC_{50} (or IC_{50}) of K, and slope of P (corresponding to the Hill coefficient, n_H). Fitting was by means of an iterative, nonlinear least squares program (21) on an Apple IIe computer. All experiments were performed in either triplicate or quadruplicate, and the results are expressed as mean ± standard deviation of the combined data of two or more experiments. A value of $p < 0.05$, as determined by Student's t test, was required for statistical significance.

Results

Effect of barbiturates on [^{3}H]acetylcholine equilibrium binding. The barbiturates studied varied in their ability to decrease equilibrium [^{3}H]acetylcholine binding to *Torpedo* membranes (Fig. 1). Secobarbital produced the smallest (0–15%), while amobarbital produced the largest (69–74%) maxi-

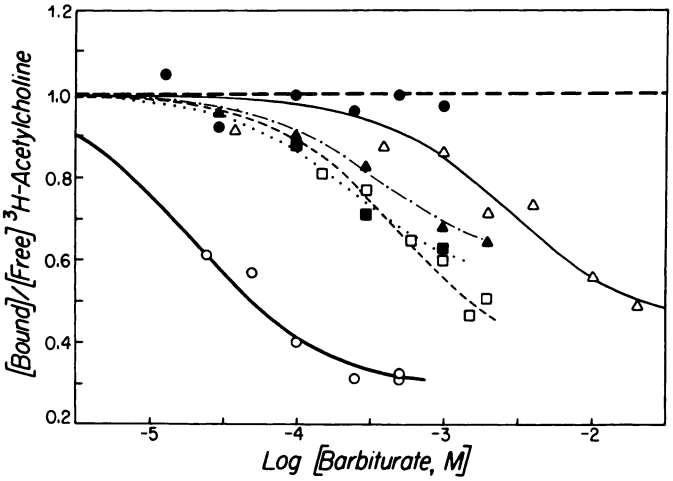

Fig. 1. Effect of barbiturates on [^{3}H]acetylcholine binding. Membranes (25–50 nM acetylcholine-binding sites), pretreated with DFP, were incubated with 25–50 nM [^{3}H]acetylcholine and varying concentrations of amobarbital (○), secobarbital (●), pentobarbital (■), phenobarbital (▲), butabarbital (□), or barbital (△) for 30 min. Maximum concentrations were limited by barbiturate solubility in *Torpedo* Ringers. Control conditions were designed for half-maximal [^{3}H]acetylcholine binding with the ratio ([Bound]/[Free] [^{3}H]acetylcholine) = 1) (30). Binding was determined by filtration as described in Experimental Procedures. The results for pentobarbital, phenobarbital, butabarbital, and barbital represent the combined data of two or more experiments. [Each point represents the mean value of triplicates (Figs. 1–3 and 5–7) or quadruplicates (Fig. 4). The standard deviation of the replicates was less than 10% of the mean value for all points in all figures, and was generally between 2% and 5%.] The results for barbital at 40 mM are off scale but were included in the data analysis (Table 1). Amobarbital and secobarbital results are representative of the more extensive data presented in Fig. 2. Concentration response curves were drawn using the logistic function with n_H = 1, the EC_{50} values in Table 1, and the best fit value of $(B/F)_{min}$ for each barbiturate (0.29 for amobarbital, 0.53 for pentobarbital, 0.57 for phenobarbital, 0.33 for butabarbital, and 0.44 for barbital).

mum decrease in [^{3}H]acetylcholine binding (with minor variations between membrane preparations). Fig. 2 illustrates a more detailed analysis of the amobarbital effect. Amobarbital decreased [^{3}H]acetylcholine binding in a concentration-dependent, saturable fashion with $EC_{50} = 20 \pm 2.4$ μM and $n_H = 1.1 \pm 0.13$ (mean ± SD of five experiments). The value of EC_{50} was unchanged when calculated with a fixed $n_H = 1$ (Table 1). Secobarbital (10^{-6}–10^{-3} M) had a minimal effect on [^{3}H]acetylcholine binding. However, secobarbital (0.25 mM) produced a significant ($p < 0.001$) rightward shift in the amobarbital concentration-response curve ($EC_{50} = 120 \pm 12$ μM, $n_H = 0.9 \pm 0.09$). Because the secobarbital data were consistent with a competitive antagonism of amobarbital, an $EC_{50} = 49 \pm 6.0$ μM was calculated for secobarbital using Eq. 2.

$$EC_{50_{amo+[seco]}} = EC_{50_{amo}} (1 + [seco]/EC_{50_{seco}}) \quad (2)$$

At higher barbiturate concentrations than those shown in Fig. 1, biphasic effects occur (16) which make it difficult to establish a plateau, especially for phenobarbital, pentobarbital, and butabarbital. However, best fit values for maximum decreases in [^{3}H]acetylcholine binding were obtained for these barbiturates as well as for amobarbital and barbital [$(B/F)_{min}$, see legend to Fig. 1]. These values permitted the estimations of EC_{50}. There were no significant differences between EC_{50} values obtained using free fit values for n_H and those using $n_H = 1$. In view of the uncertainty in $(B/F)_{min}$, fit errors for EC_{50} in Table 1 somewhat underestimate the absolute errors.

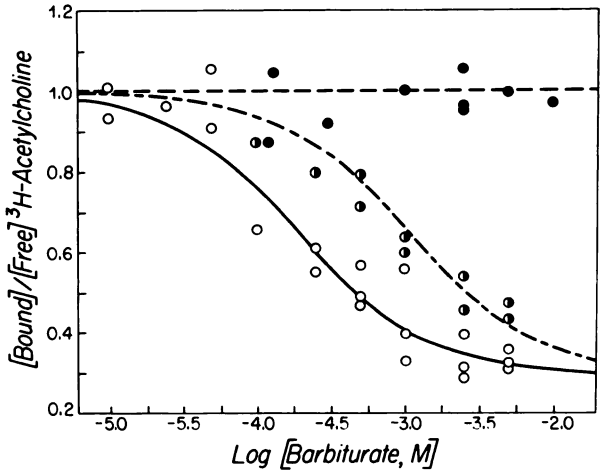

Fig. 2. Effect of amobarbital with and without secobarbital on [^{3}H] acetylcholine binding. Membranes, pretreated with DFP, were incubated with [^{3}H]acetylcholine and varying concentrations of secobarbital (●), amobarbital (○), and amobarbital with (0.25 mM) secobarbital (◑) under conditions described in Fig. 1. Binding was then determined by filtration as described in Experimental Procedures. The results presented are the combined data from three experiments for secobarbital and for amobarbital with secobarbital (0.25 mM), and the representative data from five experiments for amobarbital alone. The concentration response curves were drawn using a fixed $n_H = 1$ and the best fit value of $(B/F)_{min}$ for amobarbital [$(B/F)_{min} = 0.3$].

TABLE 1

Comparison of the abilities of barbiturates to decrease [^{3}H] acetylcholine binding (EC_{50}) and displace [^{14}C]amobarbital binding (IC_{50})

	EC_{50}[a]	(n)[b]	IC_{50}[a]	(n)
	μM		μM	
Amobarbital	20 ± 2.4	(5)	28 ± 2.9	(6)
Secobarbital	49 ± 6.0[c]	(3)	110 ± 9	(3)
Pentobarbital	230 ± 32	(2)	410 ± 46	(2)
Phenobarbital	390 ± 28	(3)	690 ± 107	(2)
Butabarbital	510 ± 54	(3)	690 ± 103	(2)
Barbital	2800 ± 650	(2)	5100 ± 910	(2)

[a] Individual binding parameters were obtained from fitting the combined data of two or more experiments to the logistic function with a fixed $n_H = 1$. Concentrations include both the un-ionized and ionized form of the barbiturate.
[b] (n), number of experiments.
[c] EC_{50} obtained from Eq. 2 (see Results).

The equilibrium binding curve for [^{3}H]acetylcholine, with and without amobarbital, was examined by centrifugation assay (Fig. 3). The equilibrium dissociation constant ($K_{eq} = 11 \pm 0.5$ nM) and Hill coefficient ($n_H = 1.5 \pm 0.08$) for [^{3}H]acetylcholine were comparable to previously published values (17–19). Amobarbital (0.2 mM) produced no change over control values in either n_H or number of binding sites (B_{max}), but did increase 3-fold the dissociation constant of [^{3}H]acetylcholine ($K_{eq} = 34 \pm 1.8$ nM, $p < 0.001$). The effect of secobarbital was examined by filtration assay. Secobarbital (0.5 mM) produced no change over control values in K_{eq}, n_H, or B_{max}.

Binding of [^{14}C]amobarbital to membranes. [^{14}C]Amobarbital binding to acetylcholine receptor-rich membranes was characterized by both a linear component and a high affinity, saturable component (Fig. 4). The linear component described [^{14}C]amobarbital binding in the presence of excess (3 mM) amobarbital and represented nondisplaceable binding. Displaceable amobarbital binding was defined as the difference between total binding and nondisplaceable binding and is plotted in Fig. 5. Displaceable binding was characterized by fitting these corrected data to the logistic function with a fixed $n_H = 1$. This yielded values for K_{eq} and B_{max} of 12 ± 3.8 μM and 370 ± 45 nM (equivalent to 1.05 μmol/g of protein), respectively (Fig. 5). The membrane suspension contained 460 nM [^{3}H] acetylcholine-binding sites (1.31 μmol/g of protein). Fitting the corrected data with assumed B_{max} values of 460 nM (stoichiometry 1:1 to acetylcholine-binding sites), 230 nM (1:2), or 920

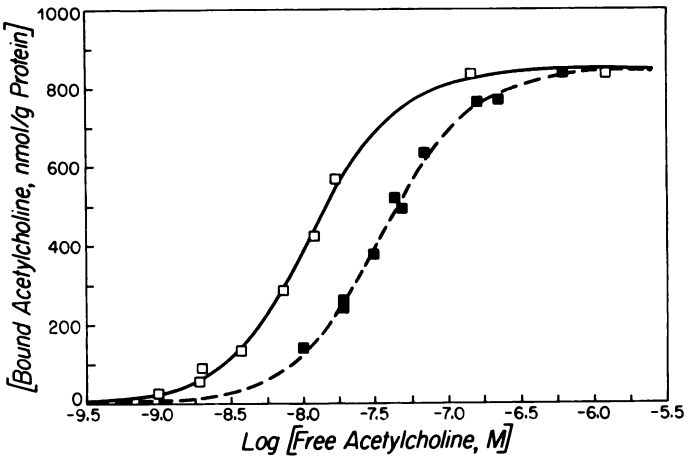

Fig. 3. Effect of amobarbital on [^{3}H]acetylcholine equilibrium binding. Membranes (0.5–0.7 μM acetylcholine-binding sites), pretreated with DFP, were incubated with varying concentrations (0.01–4.5 μM) of [^{3}H] acetylcholine with (■) and without (□) amobarbital (0.2 mM). Binding was determined by centrifugation as described in Experimental Procedures. The presence of 0.2 mM amobarbital produced no change over control values in either the Hill coefficient ($n_H = 1.4 \pm 0.10$ vs. $n_H = 1.5 \pm 0.08$) or the number of acetylcholine-binding sites ($B_{max} = 845 \pm 20$ nmol vs. 850 ± 13 nmol of acetylcholine-binding sites/g of protein) but increased the K_{eq} from 11 ± 0.5 nM to 34 ± 1.8 nM. The results represent the combined data of three experiments. The concentration curves were drawn using the best fit values for B_{max}, n_H, and K_{eq} (see above).

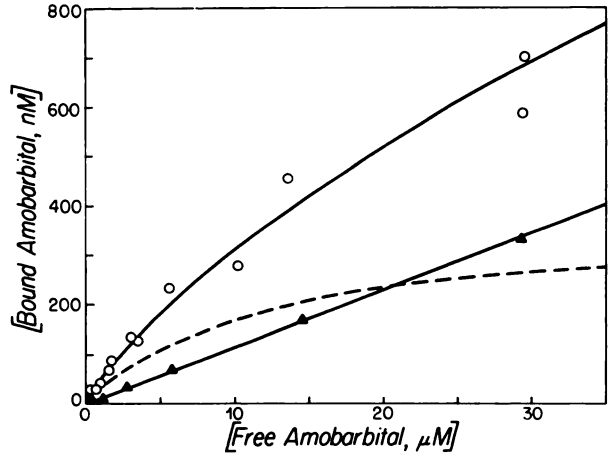

Fig. 4. Equilibrium [^{14}C]amobarbital binding. Membranes (0.5 μM acetylcholine-binding sites) were incubated with 0.7 μM [^{14}C]amobarbital and varying concentrations of unlabeled amobarbital for 30 min. Total binding (O) was then determined by centrifugation as described in Experimental Procedures. The total amount of [^{14}C]amobarbital bound to a pelleted 150-μl sample varied from 26.9 ± 0.43 nM (369 ± 10.6 cpm) in the absence of unlabeled amobarbital to 14.6 ± 0.28 nM (178 ± 2.3 cpm) in the presence of 30 μM unlabeled amobarbital. The results presented are the combined data of three experiments and, both here and below, are expressed in terms of the combined labeled and unlabeled amobarbital concentrations. Nondisplaceable binding (▲) was also determined by centrifugation assay using membranes (0.5 μM acetylcholine-binding sites) that were incubated for 30 min with amobarbital (3 mM) and varying concentrations of [^{14}C]amobarbital. The amount of [^{14}C]amobarbital nondisplaceably bound to a pelleted 150-μl sample varied from 7.0 ± 0.187 nM (108 ± 3.2 cpm) to 335 ± 4.8 nM (5170 ± 73 cpm). The results presented are the combined data of two experiments. The nondisplaceable data were fitted by linear regression through zero to give a slope = 0.0114 ± 0.00007. An equilibrium binding curve (– – –) was drawn using the data for total binding, corrected for nondisplaceable binding (see Fig. 5, *solid line*). The *curved solid line* depicting total binding was calculated from the sum of the equilibrium binding curve (K_{eq} = 12 μM) and the nondisplaceable binding.

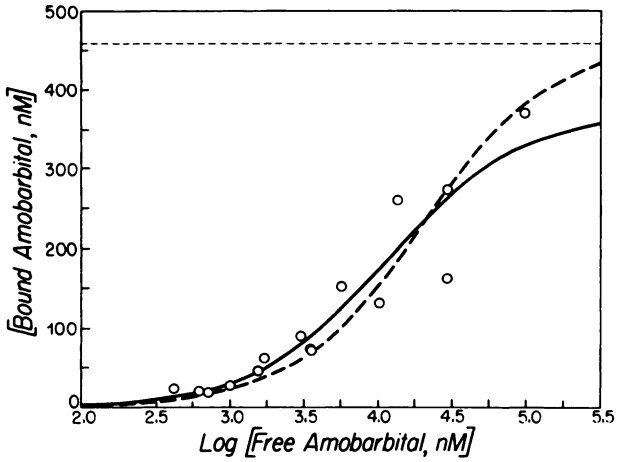

Fig. 5. Comparison of number of barbiturate-binding sites with number of acetylcholine-binding sites. The amobarbital equilibrium binding data, corrected for nondisplaceable binding, were plotted as log [free amobarbital] against [bound amobarbital]. Binding curves were drawn using either the free fit value of B_{max} (371 nM) (—) or using the number of acetylcholine-binding sites (460 nM) (– – –) as B_{max}.

nM (2:1) increased the root mean standard deviation (RMSD, a measure of goodness of fit) by 5%, 40%, and 67%, respectively, over the RMSD obtained using the free-fit value of B_{max}. Because the ratio of displaceable to nondisplaceable binding becomes very unfavorable at high occupancy, the exact stoichiometry cannot be established. However, not only is a better fit obtained with the assumption of a 1:1 stoichiometry (rather than 1:2 or 2:1), but, also, the dissociation constant is more consistent with the functional data (Table 1). However, it is not possible to entirely exclude stoichiometries of 2:1 or 1:2.

Barbiturate inhibition of displaceable [^{14}C]amobarbital binding. The effect of barbiturates on displaceable [^{14}C] amobarbital binding was examined by centrifugation assay. In a typical experiment amobarbital (1 mM) decreased [^{14}C]amobarbital binding from 480 ± 4 nmol to 190 ± 4 nmol [^{14}C] amobarbital bound/g of protein (170 ± 24 cpm to 610 ± 12 cpm) (16). Amobarbital inhibited [^{14}C]amobarbital binding in a concentration-dependent, saturable fashion, attaining a plateau in its effect between 1 and 3 mM amobarbital. (The maximum concentration possible under our experimental conditions was 3 mM.)

Inhibiton of [^{14}C]amobarbital binding was used to determine the relative affinity of the barbiturates for binding to the membrane. Both amobarbital and secobarbital inhibited [^{14}C] amobarbital binding in a concentration-dependent, saturable fashion with values for IC_{50} and n_H of 27 ± 2.9 μM, 1.1 ± 0.12 (mean ± SD, six experiments) and 110 ± 10 μM, 1.0 ± 0.09 (mean ± SD of three experiments), respectively (Fig. 6). Neither value for IC_{50} changed significantly when calculated with a fixed n_H = 1 (Table 1).

Inhibition of [^{14}C]amobarbital binding by the other four barbiturates was surveyed in a less detailed fashion. The values for IC_{50} reported in Table 1 were calculated with n_H = 1. They did not change significantly when the Hill coefficient was unconstrained.

Effect of cholinergic ligands on displaceable barbiturate binding. Carbamylcholine (100 μM) and *d*-tubocurarine

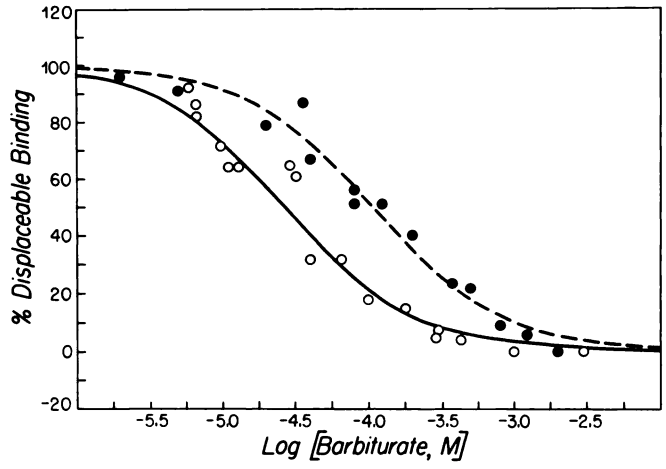

Fig. 6. Effect of amobarbital and secobarbital on displaceable [^{14}C] amobarbital binding. Membranes (4 μM acetylcholine-binding sites) were incubated with [^{14}C]amobarbital (6–7 μM) and varying concentrations of either amobarbital (O) or secobarbital (●) for 30 min. Total [^{14}C]amobarbital binding was determined by centrifugation (see Experimental Procedures). Free [^{14}C]amobarbital concentrations varied from total [^{14}C] amobarbital concentrations by less than 8%. Maximum displaceable binding was defined as the difference in total binding in the presence and absence of excess amobarbital (1 mM). The results presented are the combined data from six experiments for amobarbital and three experiments for secobarbital. The concentration response curves were drawn using the logistic function with (IC_{50} = 27 μM, n_H = 1.1) for amobarbital and (IC_{50} = 110 μM, n_H = 1.0) for secobarbital.

(100 μM) reduced total [^{14}C]amobarbital binding to acetylcholine receptor-rich membranes by 54% and 24%, respectively (Table 2). Both cholinergic-dependent reductions in total [^{14}C]amobarbital binding were blocked by preincubation with excess (11 μM) α-BTX. Excess (1 mM) amobarbital reduced [^{14}C]amobarbital binding by 58%. The remaining 42% was unaffected by either cholinergic ligand in the presence or absence of excess α-BTX. [^{14}C]Amobarbital binding alone was also unaffected by α-BTX.

This cholinergic-[^{14}C]amobarbital binding interaction was examined in more detail. Acetylcholine inhibited [^{14}C]amobarbital binding in a concentration-dependent, saturable fashion with an apparent K_{eq} = 39 ± 6.7 nM and n_H = 1.4 ± 0.30 (Fig. 7). Displaceable [^{14}C]amobarbital binding was maximally inhibited (84 ± 3.5%) by approximately 1 μM [^{3}H]acetylcholine. The remaining 16% displaceable [^{14}C]amobarbital binding was unaffected by [^{3}H]acetylcholine (10^{-6}–10^{-1} M) and was significantly less than the maximum available for displacement (84% versus 100%, $p < 0.005$). [^{3}H]*d*-Tubocurarine also inhibited [^{14}C]amobarbital binding to the receptor membrane in a concentration-dependent fashion. Displaceable [^{14}C]amobarbital binding was maximally inhibited (44 ± 5.0%) by approximately 10 μM [^{3}H]*d*-tubocurarine (Fig. 7). Unlike acetylcholine, *d*-tubocurarine binds with unequal affinity to the two acetylcholine sites on the receptor oligomer (17). Our iterative nonlinear least squares procedure would not converge when free fitting the *d*-tubocurarine data to a two-site (Adair) model. Nevertheless, the curve that was drawn from the Adair equation for two sites and using published affinity constants [K_1 = 33 nM, K_2 = 7.7 μM (17)] described the data satisfactorily (Fig. 7). Additionally, when the data were fit to the logistic function, they yielded an apparent K_{eq} = 500 ± 382 nM and n_H = 0.4 ± 0.12.

The small amount of displaceable [^{14}C]amobarbital remaining bound in the presence of cholinergic agonists (Table 2, Fig. 7) precluded its use as a radioligand for quantitative characterization of the cholinergic-induced changes in barbiturate binding constants. However, carbamylcholine (100 μM) inhibited total [^{14}C]pentobarbital binding by a maximum of 20% (15), leaving approximately 30% of the total binding capable of displacement. This 30% was just sufficient for a semiquantitative measurement of barbiturate equilibrium binding in the presence and absence of cholinergic agonists. In the absence of cholinergic agonists, both amobarbital and secobarbital displaced [^{14}C]pentobarbital (7–10 μM) binding to membranes (4–7 μM acetylcholine-binding sites) in a concentration-dependent, saturable fashion with IC_{50} values of 57 ± 12.3 μM and 220 ± 48 μM, respectively. Although carbamylcholine (100 μM) had no effect on the displacement of [^{14}C]pentobarbital by secobarbital (IC_{50} = 190 ± 48 μM), it did produce a 7-fold decrease in the apparent affinity of amobarbital (IC_{50} = 440 ± 97 μM, $p <$ 0.001). This carbamylcholine-dependent shift was in the same direction but smaller than that reported for amobarbital in displacing [^{3}H]histrionicotoxin in the presence (IC_{50} = 4 mM) and absence (IC_{50} = 24 μM) of (100 μM) carbamylcholine (22).

Discussion

Our data show that the barbiturates examined are capable, with various efficacies, of decreasing cholinergic binding to *Torpedo* acetylcholine receptor-rich membranes. Moreover, the barbiturates themselves bind to a saturable site on these membranes. A possible relationship between these findings is provided within the framework of the two-state model for cholinergic binding (23, 24).

$$\begin{array}{ccc} & \overset{K_R}{} & \\ L + R & \rightleftharpoons & LR \\ K_4 \uparrow\downarrow & & \uparrow\downarrow K_2 \\ L + D & \rightleftharpoons & LD \\ & \underset{K_D}{} & \end{array}$$

$$K_R = \frac{[L][R]}{[LR]};\ K_D = \frac{[L][D]}{[LD]};\ K_2 = \frac{[LR]}{[LD]};\ K_4 = \frac{[R]}{[D]}$$

In this model a cholinergic agonist, L, binds rapidly with dissociation constants K_R and K_D to the activatable resting state (R) and the desensitized or refractory state (D), respectively, with preferential binding to the D state ($K_D < K_R$). Despite a disregard for the transient (i.e., short-lived) open and fast desensitized receptor states, as well as the functional inequivalence and positive cooperativity between the two acetylcholine-binding sites on the receptor oligomer (25), this simplistic scheme provides an adequate description of the data.

The following arguments support a specific site model. First, [^{14}C]amobarbital binding has the following pertinent characteristics: 1) the stoichiometry is approximately two [^{14}C]amobarbital-binding sites per acetylcholine receptor oligomer (Figs. 4 and 5); 2) the site has a relatively high affinity for barbiturate binding, with an equilibrium dissociation constant (K_{eq}) for amobarbital of approximately 12 μM; 3) displaceable [^{14}C]amobarbital binding is inhibited by other barbiturates (although this inhibition could represent several different, but not independent, sites, the most economic explanation of the data is different binding affinities for the same site); and 4) cholinergic ligands inhibit amobarbital binding allosterically rather than competitively. This is consistent with the significant amount of displaceable [^{14}C]amobarbital binding unaffected by high concentrations of acetylcholine and *d*-tubocurarine (Fig. 7), as

TABLE 2

Inhibition of [^{14}C]amobarbital binding by carbamylcholine and *d*-tubocurarine

Membranes (3.5 μM acetylcholine-binding sites) were incubated with [^{14}C]amobarbital (6 μM) and either buffer, carbamylcholine (100 μM), or *d*-tubocurarine (100 μM) in the presence and absence of amobarbital (1 mM) for 30 min (total volume 3 ml). These incubations were also performed using membranes pretreated with excess (11 μM) α-BTX. Binding was determined by centrifugation assay. The results represent the means ± standard deviations of the total binding from four samples.

	Total [^{14}C]amobarbital binding			
	No α-BTX		Preincubated with α-BTX	
	Control	+1 mM Amobarbital	Control	+1 mM Amobarbital
	nmol/g protein			
Control	467 ± 8.6	195 ± 7.4	488 ± 14.0	200 ± 1.9
Carbamylcholine	217 ± 1.2	191 ± 1.2	462 ± 11.7	201 ± 5.4
d-Tubocurarine	353 ± 5.1	199 ± 4.3	473 ± 6.2	205 ± 2.7

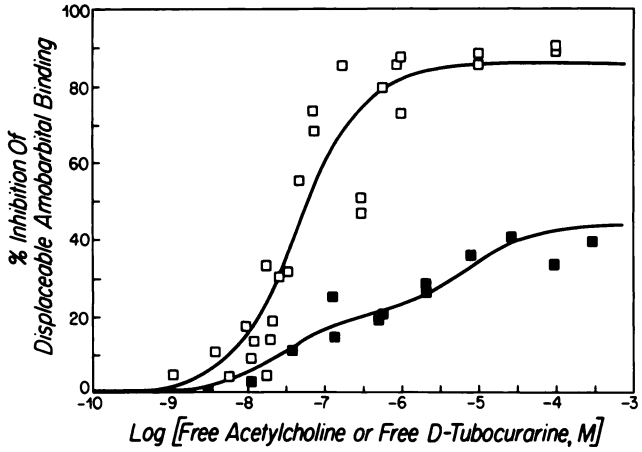

Fig. 7. Effect of acetylcholine and *d*-tubocurarine on [^{14}C]amobarbital binding. Membranes (0.5 μM acetylcholine-binding sites), pretreated with DFP, were incubated for 30 min either with [^{14}C]amobarbital (15 μM) and varying concentrations of [^{3}H]acetylcholine (□) or with [^{14}C]amobarbital (10 μM) and varying concentrations of [^{3}H]*d*-tubocurarine (■). High concentrations of [^{3}H]acetylcholine and [^{3}H]*d*-tubocurarine were serially diluted with their respective unlabeled species both to conserve radiolabeled ligands and to minimize spillover during counting. The specific radioactivity was adjusted accordingly. Binding was then determined by centrifugation (see Experimental Procedures). The results presented are the combined data (corrected for depletion) of four experiments for acetylcholine and two experiments for *d*-tubocurarine. Data obtained from free acetylcholine concentrations of 1–100 mM, which were included in the data analysis, are not presented for clarity. The curves through the data were drawn as discussed under Results.

well as with the α-BTX-cholinergic interactions with [^{14}C] amobarbital (Table 2). Note that the allosteric modulation of [^{14}C]amobarbital binding by acetylcholine (Fig. 7) occurs after preincubation with the acetylcholinesterase inhibitor DFP, again confirming that this mutual regulation involves the acetylcholine receptor rather than the esterase (15). Conversely, the effects of barbiturates on cholinergic binding have the following characteristics: 1) the barbiturate-dependent decrease in [^{3}H]acetylcholine binding is concentration dependent, saturable, and has a Hill coefficient of 1 (Figs. 1 and 2); and 2) the barbiturates interact allosterically, not competitively, with cholinergic binding, because significant amounts of [^{3}H]acetylcholine binding remain in the presence of high concentrations of barbiturates (Fig. 1). Moreover, despite its minimal effect on [^{3}H]acetylcholine binding, secobarbital apparently antagonizes the effect of amobarbital competitively (Fig. 2), implying a common allosteric site of action for both barbiturates.

Finally, the EC_{50} values, a measure of the relative ability of the barbiturates to regulate acetylcholine binding, are similar in both concentration and rank order to the IC_{50} values, a measure of their relative binding constants for the barbiturate-binding site (Table 1) [log (EC_{50}) versus log (IC_{50}), slope = 0.984, $r = 0.994$]. This finding is consistent with the hypothesis that the barbiturate-binding site and the functional site for the regulation of cholinergic binding are the same. This mutual α-BTX-dependent regulation, together with the demonstrated stoichiometry, suggests that the separate barbiturate- and acetylcholine-binding sites reside on the same receptor oligomer.

Using the two-state model, these observations can be explained by a negative heterotropic mechanism. Acetycholine has a lower affinity for the *R* state (19, 23, 24). Therefore, the ability of a barbiturate to decrease cholinergic binding reflects the relative affinity of the barbiturate for the *R* and *D* states. For example, amobarbital binds preferentially to the *R* state ($K_{R,amo} < K_{D,amo}$), whereas secobarbital binds about equally to both states ($K_{R,seco} = K_{D,seco}$). For an allosteric model such as we have assumed, with no homotropic cooperativity ($n_H = 1$), the effect of an allosteric effector, *E*, has been quantified by the change in the allosteric constant, *M′*, where

$$M' = M\,(1 + E/K_{D,E})/(1 + E/K_{R,E}) \qquad (3)$$

and $M = [D]/[R] = 1/K_4$ in the absence of agonists or effectors (26, 27). $K_{R,E}$ and $K_{D,E}$ are the dissociation constants for the effector binding to the *R* and *D* receptor states, respectively. The ability of an effector-induced change in the ratio of receptor states to change the overall binding affinity (i.e., K_{eq}) of a cholinergic agonist is defined by $K_{eq'}$ where

$$K_{eq'} = K_D \cdot K_R\,(M' + 1)/(K_R \cdot M' + K_D) \qquad (4)$$

and K_R and K_D are defined as above.

In *Torpedo* acetylcholine receptor-rich membranes, $M = 0.22$ and $K_2 \ll 1$, for acetylcholine (K_R = 800 nM and K_D = 2 nM) (19, 22). Substituting these published values and the apparent $K_{eq'}$ for acetylcholine in the presence of 15 μM [^{14}C]amobarbital ($K_{eq'}$ = 39 nM) into Eq. 4 yields a value of $M' = 0.051$. Therefore, amobarbital satisfies the model for a negative ($M' < M$) allosteric effector. (Although we have ignored the fact that $n_H \neq 1$ for cholinergic ligands, the overall conclusion is unlikely to be affected.)

In the presence of excess (100 μM) carbamylcholine, greater than 95% of *Torpedo* acetylcholine receptors will exist in the *D* state (19, 22). Therefore, amobarbital-dependent inhibition of [^{14}C]pentobarbital binding in the presence of (100 μM) carbamylcholine (IC_{50} = 440 μM) should approximate amobarbital binding to the *D* state. Substituting $K_{D,amo}$ = 440 μM and the *M′* calculated above for amobarbital into Eq. 3 yields a $K_{R,amo}$ of approximately 4 μM. As predicted by the model, the calculated $K_{R,amo}$ value is smaller than the experimentally determined apparent equilibrium dissociation constant for amobarbital binding in the absence of cholinergic ligands (K_{eq} = 12 μM). The model also predicts that for $K_{D,seco} = K_{Rseco}$, carbamylcholine will have no effect on secobarbital's inhibition of [^{14}C] pentobarbital binding. Conversely, because *d*-tubocurarine is less effective than carbamylcholine and acetylcholine in stabilizing the *D* state (28, 29), it will have less effect on amobarbital binding. Therefore, the data are both qualitatively and semiquantitatively consistent with the two-state allosteric model for barbiturate-cholinergic interactions.

TABLE 3

Comparison of the relative potencies of two barbiturate effects

The ability of the barbiturates to bind to the acetylcholine receptor (IC_{50}, Table 1) is compared to their ability to induce general anesthesia, as measured by loss of righting reflex (ED_{50}-LRR) (33). These two effects are also compared to their lipid solubility [log *P*, where *P* is the octanol/water partition coefficient (3, 38)] (see Discussion). Table 3 represents the concentration of the unionized species [pK values were taken from a recent compilation (38)].

Compound	IC_{50}	ED_{50}-LRR	Log *P*
	μM	μM	
Amobarbital	24	336	2.23
Secobarbital	98	67	2.37
Pentobarbital	366	135	2.23
Phenobarbital	435	1,640	1.42
Butabarbital	627	470	1.65
Barbital	4,300	11,108	0.72

Many other agents are known to modulate acetylcholine binding by interacting with specific allosteric sites on the acetylcholine receptor oligomer (13, 22, 27, 29–32), with local anesthetics the most thoroughly characterized group. Most of these agents enhance acetylcholine binding, but this is by no means a universal property. For example, tetracaine decreases acetylcholine binding (22, 31), a property shared with the benzomorphan opiates (32). There is more than one class of sites for local anesthetic binding on the acetylcholine receptor (13), but a structurally diverse group of agents apparently interacts with the high affinity site labeled by histrionicotoxin and phencyclidine (22, 32). Although amobarbital inhibits [^{3}H] histrionicotoxin binding (22), extensive studies are required to distinguish between competitive interactions at a single allosteric site and allosteric interactions among one or more such sites. These studies will be difficult because of the problems entailed in determining the concentration-response relationships of compounds with limited aqueous solubility (Figs. 1 and 2, for example).

We also have compared the ability of the barbiturates to bind to the acetylcholine receptor (IC_{50}) in relation to their ability to induce general anesthesia, as measured by the ED_{50} for loss of righting reflex (33) (Table 3). As determined by linear regression, barbiturate binding does not correlate as well with lipid solubility [log (IC_{50}) versus log P, $r = -0.852$] as does anesthetic potency [log (ED_{50}-LRR) versus log P, $r = -0.967$]. More importantly, the IC_{50} does not correlate with ED_{50}-LRR [log (IC_{50}) versus log (ED_{50}-LRR), slope = 0.688, $r = 0.720$], differing in both magnitude and rank order. However, anesthesia does occur at barbiturate concentrations that would cause between 36% and 95% of the barbiturate receptors on the acetylcholine receptor to be occupied. In the case of amobarbital, half-occupancy occurs at a little below one-tenth of the anesthetic concentration. The sensitivity of the neuronal acetylcholine receptor is, thus, of great interest.

Allosteric regulation of ligand binding by barbiturates has been noted in other systems (8, 34–37). This subject has been most extensively studied in the GABA receptor-ionophore complex. In our research the IC_{50} values for displacing [^{14}C]amobarbital were similar to those reported for displacing [^{3}H]-α-dihydropicrotoxinin and caged convulsants from the GABA-receptor complex (5–9, 34). The parallels between the barbiturate action on the acetylcholine receptor and the GABA receptor are quite extensive (Refs. 5–9 compared with Refs. 10–13, 16, 17, 19, and 22). However, our results differ from those on the GABA receptor both in rank order and in the stereoselective effect of pentobarbital (15). Although the barbiturate sites on both GABA and acetylcholine receptors are allosteric sites, these findings suggest that they have significant structural differences.

We have presented evidence that the barbiturates bind with moderate affinity (10^{-5}–10^{-3} M) to allosteric sites on the acetylcholine receptor oligomer. There appears to be roughly one barbiturate-binding site per acetylcholine-binding site, and these sites are allosterically coupled to each other. Within the limitations of our experimental results, a two-state model provides a sufficient mechanism of action. In this model, the degree of negative heterotropic interaction between barbiturates and cholinergic agonists is dependent upon the relative affinity of a particular ligand for binding to the activatable rather than to the desensitized receptor state. Further studies are necessary to determine the relationship between this barbiturate site and other allosteric sites reported on the acetylcholine receptor.

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