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Actions of Pentobarbital Enantiomers on Nicotinic Cholinergic Receptors

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SUMMARY

The enantiomers of pentobarbital had four different actions on the nicotinic receptor-rich membranes from *Torpedo* electroplaques. (i) Both inhibited cholinergically stimulated cation flux through the receptor's channel, with IC_{50} values of ~25 μM and extremely weak stereoselectivity. (ii) (*R*)-(+)-[^{14}C]Pentobarbital bound to a saturable site with an apparent dissociation constant of 100 μM, a Hill coefficient of 1.2, and a stoichiometry of 1:1 with the acetylcholine binding sites. (*S*)-(−)-Pentobarbital also displaced (+)-[^{14}C]pentobarbital but its IC_{50} was 4-fold higher than that of the (+)-enantiomer under the same conditions. (iii) Both enantiomers caused a stereoselective allosteric inhibition of [^{3}H]acetylcholine binding, which occurred over the same concentration range and with the same stereoselectivity as barbiturate binding. (iv) Above 1 mM, pentobarbital caused an unexpected and sudden increase in [^{3}H]acetylcholine binding, which lacked significant stereoselectivity. These results are consistent with a model where low concentrations of pentobarbital act on the receptor by binding to allosteric sites that have higher affinity but lower stereoselectivity for the open channel conformation than for the resting conformation, whereas the highest concentrations of pentobarbital act by nonspecific mechanisms mediated by general membrane perturbations.

Barbiturates, as a class of compounds, exert a broad spectrum of pharmacological actions on excitable membrane properties and synaptic transmission (1–3). In particular, the distinct physiological actions that pentobarbital can produce in a variety of systems have been well documented. Major effects include potentiation (and even activation) of GABA receptor-mediated inhibitory currents, depression of voltage-activated calcium conductances, and inhibition of acetylcholine-activated excitatory currents (for reviews see Refs. 4–8). All of these effects may contribute to depression of overall electrical excitability (9), but it remains unresolved whether such depression is the primary contributory factor to barbiturate anesthesia (10).

Barbiturate actions may have several underlying mechanisms ranging from nonspecific membrane perturbations to specific interactions with multifunctional sites and/or receptors (11). Among the tools useful for distinguishing between these various actions and elucidating their underlying mechanisms is the stereoselectivity of enantiomeric barbiturates. Stereoselectivity of barbiturate action has been demonstrated *in vitro* at the GABA receptor complex (4, 8, 12), the glutamate receptor (4), the isolated crayfish stretch receptor (2), the CA1 and dentate neurons of the hippocampus (9, 10), the calcium-dependent action potential (9), and the nicotinic receptor from *Torpedo* (13), among others. In some cases the observed actions were not only stereoselective but biphasic as well, suggesting multiple modes of action and/or differential actions on different conformations of the same macromolecule (4, 14, 15).

Because barbiturates may exert multiple actions at each of several targets, it would be advantageous to study their action in a well characterized preparation containing a homogeneous population of receptors. Such a system is provided by the nicotinic receptor-rich membranes isolated from *Torpedo* electroplaques. In these membranes, some 25% of the total protein may be acetylcholine receptors, a specific activity some 4 orders of magnitude higher than that achievable in postsynaptic membrane preparations from the central nervous system. This high receptor density allows more detailed mechanistic studies to be performed. Thus, in these membranes we were able to directly demonstrate a binding site for radiolabeled barbiturates that exhibited stereoselectivity for the enantiomers of pentobarbital

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ABBREVIATIONS: GABA, γ-aminobutyric acid; TBPT, *t*-butylbicyclophosphorothionate.

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(13) and that interacted allosterically with the cholinergic site (16). Here we characterize this stereoselective binding in greater detail, comparing it with the functional effects of the pentobarbital enantiomers on [^{3}H]acetylcholine binding and on inhibition of cholinergically stimulated ion flux.

Experimental Procedures

Materials

[^{3}H]Acetylcholine (1.02 mCi mmol^{-1}) was from Amersham Corp. (Arlington Heights, IL); *Torpedo nobiliana* from Biofish Associates (Georgetown, MA); diethyl ethyl-(+)-2-pentylmalonate from Aldrich Chemical Co. Inc. (Milwaukee, WI); [^{14}C]urea (56 mCi mmol^{-1}) from New England Nuclear (Boston, MA); polypropylene microcentrifuge tubes from VWR Scientific Inc., (Boston, MA); Versi Scint I from Fisher Scientific (Pittsburgh, PA); and Sephadex G-50 from Pharmacia Fine Chemicals (Piscataway, NJ).

Methods

Membrane preparation. Membranes, enriched in acetylcholine receptor, were prepared as previously described (16) from freshly dissected. *T. nobiliana* electroplaques, using differential and sucrose gradient centrifugation techniques. These membranes were kept at −80° until required. The specific activity of the acetylcholine sites per gram of protein was further enhanced by a final velocity gradient step (17), and these membranes were stored under nitrogen at 4° for up to 2 weeks. Protein was determined by the method of Hartree (18) with bovine serum albumin as standard.

Synthesis of (*R*)-(+)-[^{14}C]pentobarbituric acid. The radiolabeled (+)-[^{14}C]pentobarbituric acid was synthesized, according to the methods of Cooke and Talant (19), from diethyl ethyl-(+)-2-pentylmalonate and [^{14}C]urea (56 mCi mmol^{-1}) and was purified by thin layer chromatography. The final product, approximately 50 μg, was stored in 0.2 ml of benzene. Just before the series of experiments, the benzene was removed by evaporation under a stream of nitrogen and replaced with 13.8 ml of ethyl alcohol. The final ethanolic solution was stored in a glass vial at −40°.

Enantiomers of pentobarbital. The enantiomers of pentobarbital were either prepared as above [(+)-enantiomer] or were a gift from the National Institute of Drug Abuse [(+)- and (−)-enantiomers]. Concentrated solutions of the enantiomers of pentobarbituric acid were initially dissolved in calcium-free *Torpedo* Ringer's solution made alkaline with 1 N NaOH. $CaCl_2$ was then added, and the pH was immediately adjusted to 7.0 using 1 N HCl. All solutions were prepared fresh daily before each experiment. Appropriate dilutions of the stock solutions were made using *Torpedo* Ringer's solution (NaCl, 250 mM; KCl, 5 mM; $CaCl_2$, 3 mM; $MgCl_2$, 2 mM; NaN_3, 0.02%, and Na_2PO_4/$NaHPO_4$, 5 mM) at pH 7.0.

(+)-[^{14}C]Pentobarbital binding assay. The methods for the radiolabeled binding and displacement studies were similar to those previously described (13). The binding of (+)-[^{14}C]pentobarbital was assayed in *Torpedo* Ringer's solution at room temperature, pH 7.0. An aliquot of the ethanolic solution of (+)-[^{14}C]pentobarbital was dried under nitrogen in a 1.5-ml polypropylene microcentrifuge tube. When dry, a sufficient quantity of membrane suspension was added, mixed gently on a Vortex, and allowed to incubate for at least 30 min with gentle mixing at 10-min intervals. Fifty-microliter aliquots of this (+)-[^{14}C]pentobarbital-containing membrane suspension were transferred to other 1.5-ml polypropylene capped microcentrifuge tubes and 350-μl aliquots of *Torpedo* Ringer's solution, with or without drug, were added as desired. The contents of each microcentrifuge tube were mixed gently on a Vortex mixer and allowed to equilibrate, with frequent mixing, for a period of at least 30–45 min. Following the incubation period, three 100-μl aliquots from each tube were transferred to microcentrifuge tubes and centrifuged for 30 min at 135,000 × *g* in a Beckman Airfuge to yield a tight pellet. The resulting supernatants were removed by micropipette and stored in 1.0-ml capped polypropylene vials. When the procedure below was complete, 50-μl aliquots of each supernatant were transferred to scintillation vials containing 6 ml of scintillation fluid. The pellet was immediately washed using three 150-μl aliquots of ice cold *Torpedo* Ringer's solution (the wash solution was pipetted into and out of the centrifuge tube in one smooth motion). To solubilize the pellet, a 100-μl aliquot of 10% sodium dodecyl sulfate was added to each tube and the tubes were heated in an oven at 50–80° for 1 hr. The complete centrifuge tube was transferred into a scintillation vial containing 6 ml of Versi Scint I. All samples were counted in a Beckman LS 8000 scintillation counter to a standard deviation of 1.0%.

[^{3}H]Acetylcholine binding. The binding of [^{3}H]acetylcholine to acetylcholine receptor-rich membranes in the presence and absence of pentobarbital enantiomers was performed using centrifugation, as previously described (16). Briefly, membranes (20 nM acetylcholine binding sites), preincubated with diisopropylfluorophosphate to inactivate acetylcholinesterase, were incubated with [^{3}H]acetylcholine (20 nM) with or without pentobarbital for at least 15 min, after which they were centrifuged on a Beckman Airfuge as in the barbiturate assay above.

Agonist-induced cation flux. Flux was measured as described previously (20). Briefly, net efflux of $^{86}Rb^+$ from sealed vesicles at 4° was measured after a 10-sec exposure to agonist. ^{86}RbCl (100 to 200 μCi/ml), vesicles (3 to 7 μM in acetylcholine sites), and desired stoichiometric amounts of α-bungarotoxin were incubated overnight at 4°. Extravascular $^{86}Rb^+$ was first removed by exclusion chromatography (Sephadex G-50, 0.5 cm × 20 cm), and again 20 min later by rapid passage over a cation exchange column (Dowex 50W, 20–50 mesh). Efflux was initiated when an aliquot of $^{86}Rb^+$-loaded vesicles was mixed with buffer containing agonist, and pentobarbital where appropriate, (final acetylcholine receptor concentration was ≤50 nM). After 10 sec, the mixture (1 ml) was filtered through Whatman GF/F filters in a vacuum manifold. The filtrate (0.5 ml) was mixed with scintillation cocktail and counted. Slow, time-dependent leakage of $^{86}Rb^+$ from vesicles was measured from filtrates of experiments without agonist at intervals after elution from the ion exchange column. A linear least-squares fit of filtrate counts versus time gave an estimate of leakage rate, $cpm_{leak,t}$, at the time of filtration for experiments done in the presence of agonist. Total counts, cpm_{total}, were measured from unfiltered vesicle solutions. Time-dependent efflux response, F_a, is reported as the percentage of non-leak $^{86}Rb^+$ counts released:

$$F_a = \frac{cpm_{Ag,t} - cpm_{leak,t}}{cpm_{total} - cpm_{leak,t}} \times 100\% \quad (1)$$

Analysis of results. The concentration dependence of each effect could usually be fit to a logistic function of the form

$$E = E_{max}\left(\frac{[PB]^n}{[PB]^n + K^n}\right) \quad (2)$$

where E is any effect (for example binding or flux), E_{max} is its maximum value, $[PB]$ is the concentration of pentobarbital in buffer, K is the half-effect concentration (for example IC_{50}), and n is the slope parameter corresponding to the Hill coefficient (21).

Results

Stereoselectivity of displaceable (*R*)-(+)-[^{14}C]pentobarbital binding. Typically, when membranes were equilibrated with 2–3 μM (+)-[^{14}C]pentobarbital, the total pentobarbital in the pellet was 180–200 nmol/g of protein. This was reduced to about 130 nmol/g of protein by addition of high concentrations (4.5 mM) of either (±)- or (+)-pentobarbital. The difference in total (+)-[^{14}C]pentobarbital in the pellet in the absence and presence of excess pentobarbital was defined as displaceable binding. The standard deviation of the total

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binding (determined in triplicates) averaged about 2.5% and resulted in combined errors in displaceable binding of about 10%. The percentage of the (+)-[^{14}C]pentobarbital displaced is plotted as a function of total unlabeled (+)- or (−)-pentobarbital concentration in Fig. 1. When analyzed by nonlinear least squares, the (+)-pentobarbital data yielded an IC_{50} of 130 ± 15 μM (mean ± SD) and a Hill coefficient of 0.94 ± 0.11, whereas for (−)-pentobarbital the IC_{50} and Hill coefficient were 525 ± 46 μM and 1.25 ± 0.14, respectively. The ratio of the IC_{50} values of the enantiomers is 4.0 ± 0.59, indicating a modest but significant stereoselectivity, consistent with preliminary estimates by Miller *et al.* (13).

Stoichiometry of displaceable (+)-[^{14}C]pentobarbital binding. The data in Fig. 1 were recalculated, correcting for dilution of the specific radioactivity, to yield the actual concentration of (+)-pentobarbital bound to the receptor. An unconstrained nonlinear least squares fit of this binding to Eq. 2 yielded a dissociation constant of 100 ± 35 μM, a Hill coefficient of 1.21 ± 0.046, and a stoichiometry of 2.4 ± 0.40 mmol of binding sites/kg of protein. The same membrane preparation contained 1.9 ± 0.30 mmol of [^{3}H]acetylcholine binding sites/kg of protein, yielding a stoichiometry of 1.3 ± 0.28 pentobarbital sites/acetylcholine site, not significantly different from a stoichiometry of 1 to 1. Two previous estimates of stoichiometry, which were obtained with [^{14}C]amobarbital (16) and with (±)-[^{14}C]pentobarbital (13) and yielded values of 0.8 and 1.4, respectively, are in satisfactory agreement, considering the difficulties imposed by the unfavorable ratio of displaceable to nondisplaceable binding.

Biphasic modulation of [^{3}H]acetylcholine binding. When barbiturates bind to their site on the acetylcholine receptor, they modulate the binding of acetylcholine (13, 16). In these experiments, about half the acetylcholine receptors were occupied by [^{3}H]acetylcholine, so that barbiturate-induced increases or decreases in binding could be readily detected. The results were expressed as the change in the ratio of the bound to the free [^{3}H]acetylcholine concentration. In the present experiments (Fig. 2), both enantiomers gradually decreased acetylcholine binding, with (+)-pentobarbital causing a greater decrease at a given concentration than (−)-pentobarbital. However, at 1.5–3 mM this change reversed directions and there occurred a sharp and significant ($p < 0.01$) increase in the ratio of bound to free acetylcholine concentration that continued, without saturating, up to the highest pentobarbital concentrations we could examine, where the levels of acetylcholine binding attained were comparable to those of the controls. This biphasic effect was consistently observed only when care was taken to keep the pentobarbital in solution (see Experimental Procedures).

To further characterize the decrease in acetylcholine binding, we examined the effect of racemic pentobarbital on the binding curve of acetylcholine. The concentration of acetylcholine for half-maximum binding (EC_{50}) was increased by 1 mM pentobarbital from 5.4 ± 0.65 to 9 ± 2.7 nM and the Hill coefficient remained unchanged (1.48 ± 0.076 versus 1.6 ± 0.24). Pentobarbital causes a similar rightward shift of the binding curve of *d*-[^{3}H]-tubocurarine (22), as does amobarbital on the binding curve of [^{3}H]acetylcholine (16).

Stereoselectivity and the modulation of [^{3}H]acetylcholine binding. The modulation of [^{3}H]acetylcholine binding by pentobarbital appeared stereoselective at low, but not at high, concentrations. The difference between the two enantiomers in decreasing acetylcholine binding became significant (p

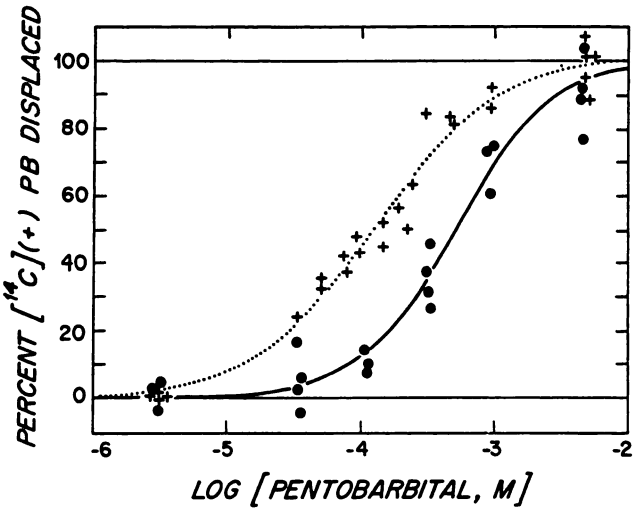

Fig. 1. Displacement of (+)-[^{14}C]pentobarbital by the enantiomers of pentobarbital. Five separate experiments are represented and each *point* is the mean of three samples (see Experimental Procedures for experimental details). +, (+)-pentobarbital; ●, (−)-pentobarbital. The *lines* were fit by nonlinear least squares to Eq. 2, which yielded IC_{50} values and Hill coefficients (or slopes), respectively, of 130 ± 15 μM (mean ± SD) and 0.9 ± 0.11 for (+)-pentobarbital and 525 ± 46 μM and 1.25 ± 0.14 (−)-pentobarbital. The free (+)-[^{14}C]pentobarbital concentration was ≤3 μM.

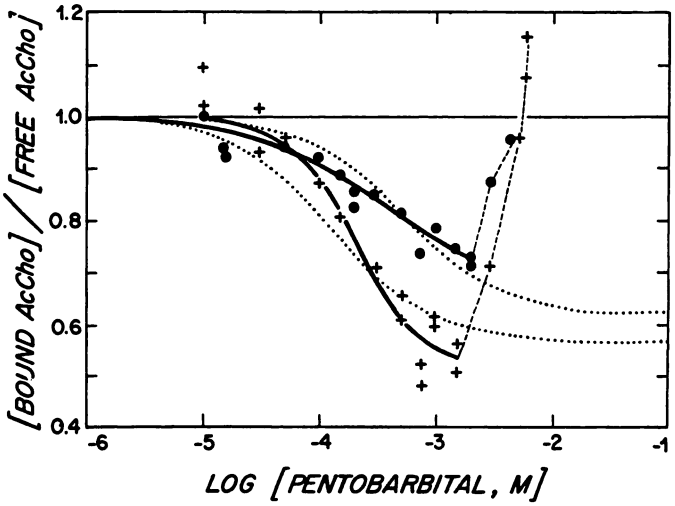

Fig. 2. Biphasic modulation of [^{3}H]acetylcholine binding by the enantiomers of pentobarbital. Both the (−)- (●) and the (+)- (+) enantiomers of pentobarbital cause a decrease in [^{3}H]acetylcholine binding at low concentrations and an increase at higher concentrations. The total number of points was 40 and 62 for the (+)- and (−)-enantiomers, respectively. Each *point* is the mean of three to six determinations, except where two points are shown at one concentration, when they represent individual determinations. *Error bars* are omitted for clarity. The average SD was 0.05 for the (+)- and 0.08 for the (−)-enantiomer, respectively. Data points at the highest concentrations deviate from the trend and have simply been joined by *dotted lines*. The remaining points in each set were fitted by nonlinear least squares to Eq. 2 in two ways (see Results). The *solid curves* are the best fits when the slope, IC_{50} and minimum value of the *y*-axis are all variables. The parameters determined are given in the text. The *dashed curves* were fitted by nonlinear least squares assuming that acetylcholine is displaced as a result of binding to the barbiturate binding site studied in Fig. 1. The Hill coefficients were fixed equal to 1 and the dissociation constants (or IC_{50} values) to the values in the legend to Fig. 1.

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< 0.01) at 150 μM and reached a maximum ratio of 1.35 ± 0.16 at 1–1.5 mM. Above this concentration, the difference between the enantiomers declined and was lost by 5 mM.

The observed decrease in acetylcholine binding does not plateau convincingly before it increases again and, therefore, it is uncertain whether the stereoselectivity of pentobarbital arises because the enantiomers differ in efficacy or in potency. In an attempt to resolve this question, we analyzed the decrease in acetylcholine binding by excluding data above 1.5 mM for (+)-pentobarbital and above 3 mM for (−)-pentobarbital and then adopting two strategies. First, we fitted the remaining data by nonlinear least squares to Eq. 2 (Fig. 2, *heavy lines*). The minimum value of bound/free acetylcholine for (+)-pentobarbital extrapolated to 0.51 ± 0.037 and for (−)-pentobarbital to 0.7 ± 0.12, a difference of 0.2 ± 0.13. A second estimate of the maximum effect was obtained by assuming a model of noninteracting sites with affinities given by the IC_{50} values obtained from displacing (+)-[^{14}C]pentobarbital (Fig. 2, *dotted lines*). This model fits the data reasonably satisfactorily, with only small systematic deviations of the data from the lines, and yields minimum values for bound/free acetylcholine of 0.56 ± 0.021 and 0.62 ± 0.024 for the (+)- and (−)-enantiomers, respectively. The difference of 0.06 ± 0.032 is smaller than our estimated errors. These two estimates are in reasonable agreement and suggest that there is little or no stereoselectivity in the efficacy of the action of pentobarbital. The difference in the two sets of data must then largely reflect a difference in affinity. The first strategy adopted above yielded values of 210 ± 35 and 320 ± 320 μM for the (+)- and (−)-enantiomers, respectively (*heavy lines* in Fig. 2), where the large inherent errors obscure the stereoselectivity. However, the second model shows that the data are consistent with the hypothesis that the decrease in [^{3}H]acetylcholine binding occurs as a result of the occupation of the barbiturate bindings sites that are detectable by (+)-[^{14}C]pentobarbital binding experiments (Fig. 1).

Inhibition of carbachol-induced cation flux. The concentration dependence of inhibition of the maximum flux elicited by carbachol (5 mM) was studied with racemic pentobarbital and with both enantiomers (Fig. 3). The background leak of $^{86}Rb^{+}$ from the vesicles was not altered by pentobarbital concentrations up to 2 mM. In all cases the concentration dependence of inhibition could be fit to Eq. 2. In two independent experiments on racemic pentobarbital using different batches of receptor, the IC_{50} values were 25.1 ± 0.90 and 21 ± 1.8 μM and the Hill coefficients were 0.95 ± 0.033 and 1.3 ± 0.13, respectively. The enantiomers inhibited with identical Hill coefficients of 1.25 ± 0.08 and IC_{50} values of 21 ± 1.2 and 30 ± 1.8 μM for the (+)- and (−)-enantiomers, respectively. The difference of 9 ± 2.1 μM was significant ($p < 0.001$), yielding a stereoselectivity ratio of 1.5 ± 0.10. In a second experiment we found a difference of 7 ± 2.6 μM ($p < 0.001$) and a ratio of 1.20 ± 0.084. Thus, in each experiment the (+)-enantiomer was consistently and significantly more potent than the (−)-enantiomer, but the size of this effect was small compared with our errors between experiments.

The effect of pentobarbital on the carbachol concentration-flux curve was studied at a single concentration of pentobarbital (14.4 μM). The (+)- and (−)-enantiomers behaved similarly, shifting the EC_{50} of carbachol from 120 ± 19 μM to 190 ± 24 and 170 ± 28 μM, respectively, and lowering the maximum flux by around 40%. These observations are consistent with a previous report that racemic pentobarbital acts as a noncompetitive inhibitor of carbachol-induced cation flux, lowering the maximum effect of the agonist while shifting its half-effect concentration about 20% to the right, a small deviation from predicted behavior that probably reflects the role of desensitization during the 10-sec flux assay (23).

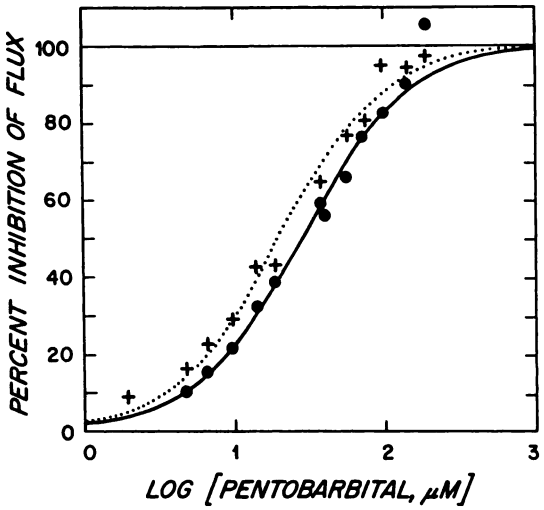

Fig. 3. Inhibition of cholinergically stimulated cation flux. Maximal $^{86}Rb^{+}$ efflux was elicited by exposure to 5 mM carbachol for 10 sec. Pentobarbital, in appropriate concentrations, was added simultaneously with the carbachol. Results for a single experiment are shown; each *point* represents a single determination. The *curves* are from nonlinear least squares fitting of the data to Eq. 2. For (+)-pentobarbital (+) the parameters were IC_{50} = 20 ± 1.2 μM and Hill coefficient = 1.25 ± 0.080 and for (−)-pentobarbital, (●) they were 30 ± 1.8 μM and 1.25 ± 0.88, respectively.

Discussion

Model of the action of pentobarbital. It is well known that the acetylcholine receptor from *Torpedo* electroplaques can exist in several interconvertible conformations or states. To help simplify a detailed discussion we have cartooned the action of pentobarbital in Fig. 4, which shows an adaptation of the conventional scheme (for a review see Ref. 24). At rest, in the absence of either agonist or pentobarbital, about 80% of the receptors are in the resting state (state 1 in Fig. 4), which has a low affinity for acetylcholine, and the remaining 20% of the receptors are in a desensitized state (state 3 in Fig. 4), which has a high affinity for acetylcholine (25). Agonist binding rapidly converts the resting conformation to the open state (state 2), probably in several steps (not shown). Activity is terminated by fast desensitization within seconds (not shown) or by dissociation of the agonist and reversion to the resting state.

The simplest hypothesis that accounts for our results is that pentobarbital acts both by binding to an allosteric site whose affinity varies with the conformational state of the acetylcholine receptor and by a nonspecific action on the receptor's surrounding lipids. Within this scheme, pentobarbital binds with the highest affinity (~10^{-5} M) to the open state (transition 2 → 5), corresponding to the observed inhibition of ion flux with IC_{50} of ~25 μM. At somewhat higher concentrations, pentobarbital binds with lower affinity (~10^{-4} M) to the resting state of the receptor (transition 1 → 4), whose two identical sites (shown arbitrarily as distinct from the channel block site; see below) exhibit 4-fold stereoselectivity for the (+)-enan-

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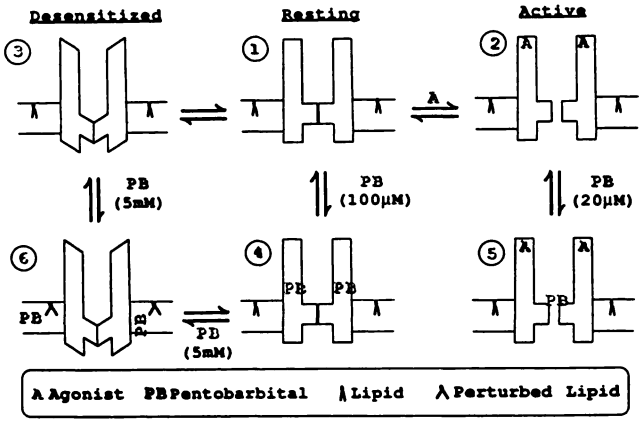

Fig. 4. Schematic illustration of the three actions of pentobarbital on acetylcholine receptors. The receptor, shown in cross-section in its membrane environment (45), is illustrated as having three states, resting (state 1), activated (state 2), and desensitized (state 3). Pentobarbital has different affinities for each of these states (*bottom row*), binding with the highest affinity to the activated state (state 5), with moderate affinity to the resting state (state 4), and with negligible affinity to the desensitized state. The model assumes that binding to these sites is conformationally selective but does not in itself induce new conformations; this assumption is based on work on local anesthetics reviewed in Ref. 24.

tiomer. This binding reduces the proportion of receptors in the desensitized state (transition 3 → 1), resulting in an allosteric and stereoselective reduction in acetylcholine binding. However, even higher concentrations of pentobarbital (>10^{-3} M) disorder the lipid bilayer of the membrane, thus stabilizing the desensitized state (transition 4 → 6) and accounting for the observed sharp increase in acetylcholine binding.

Physiological relevance. In order to put these effects of pentobarbital in physiological perspective, they may be compared with the general anesthetic potency of pentobarbital. The ED_{50} in amphibians at this temperature has been found to be 150–300 μM (reviewed in Ref. 26). At this concentration, ion flux inhibition of pentobarbital would be in the range of 83–91% and the occupancy of the pentobarbital binding site by the (+)-enantiomer would be in the range of 53–70%. However, the occupancy by the (−)-enantiomer would be only 22–36%. It is thought that the (−)-enantiomer is more potent than the (+)-enantiomer in causing general anesthesia in mammals, although the possibility of differential pharmacokinetics leaves some uncertainty (reviewed in Ref. 6). Thus, in agreement with our previous work (16), there is no correlation between anesthetic potency and binding to this allosteric site.

Ion channel inhibition by pentobarbital. Pentobarbital inhibits cholinergically stimulated ion flux noncompetitively, but our experiments were carried out over too long a time scale to define precisely the underlying mechanism. Nonetheless, previous work (20) shows that, for many anesthetics, inhibition curves determined on a 10-sec time scale are comparable to those determined on a millisecond time scale because any open channel is rapidly blocked. Pentobarbital was equally effective as an inhibitor whether we preincubated the membranes with it or added it simultaneously with the antagonist. Thus, it too probably acts rapidly, a conclusion supported by electrophysiological studies that suggest that pentobarbital binds preferentially to the open state of the receptor (27, 28).

It appears that pentobarbital acts by binding to more than one site on the acetylcholine receptor, because the inhibition curves had Hill coefficients of 1.25. Although the lack of marked stereoselectivity, which is consistent with electrophysiological experiments (4), might argue against a discrete site of action, the concentrations effective at inhibiting flux are much lower than those required to perturb the membrane (23). Indeed, studies of acetylcholine-induced sodium currents in *Aplysia* provide indirect evidence for a barbiturate site (29).

Allosteric interactions between barbiturate and acetylcholine sites. In the absence of channel activation, we have shown previously that pentobarbital binds preferentially to the resting state, increasing the proportion of the receptors in this state (states 1 and 4 in Fig. 4) and decreasing the proportion in the desensitized state (state 3 and 6 in Fig. 4) (13, 16). Because acetylcholine binds with higher affinity to the desensitized state than the resting state, this results in a mutual negative heterotropic interaction between the barbiturate and cholinergic sites. That is, [^{14}C]barbiturate binding is reduced by acetylcholine and [^{3}H]acetylcholine binding is reduced by barbiturates (16). Consistent with this model, our present data show that the binding isotherms for both enantiomers follow, within the experimental uncertainties, the concentration-response curves for decreasing [^{3}H]acetylcholine binding (Fig. 2).

Desensitizing action of pentobarbital. As the pentobarbital concentration is increased further, [^{3}H]acetylcholine binding suddenly stops decreasing and then begins to climb steeply (Fig. 2), suggesting that an increasing proportion of the acetylcholine receptors assume the desensitized conformation, which has the highest affinity for acetylcholine (transition 4 → 6 in Fig. 4). Solubility limitations prevented a complete study, but the steepness of the concentration-effect curves and the lack of stereoselectivity are reminiscent of the behavior of the alcohols and volatile anesthetics. These agents all (i) increase [^{3}H]acetylcholine binding with a very steep concentration dependence, typically exhibiting Hill coefficients of 2 to 3 (30); (ii) stabilize the desensitized state of the receptor (22, 31, 32); and (iii) act without stereoselectivity (33).

On the basis of experiments in which the acetylcholine receptor was reconsitituted into bilayers of various lipids, it has been proposed that an optimal fluidity is necessary to prevent desensitization (34). The mechanism of receptor desensitization induced by alcohol and volatile anesthetics is also thought to be indirect, mediated by a nonspecific perturbation of the lipid bilayer surrounding the acetylcholine receptor. Indeed, the desensitizing potency of these agents correlates with their ability to disorder the lipid of the acetylcholine receptor-rich membranes (23) and their action is reversible by high pressure (35). Also, enantiomers of general anesthetics perturb lipid bilayers equally (33, 36). The mechanism of the pentobarbital-induced desensitization requires further study, but preliminary data indicate a mechanism similar to that of the above agents is possible (23).

Stereoselectivity of the binding site. It is somewhat puzzling that the pentobarbital site that is postulated to inhibit agonist-stimulated cation flux exhibits higher affinity but weaker stereoselectivity than the site in the resting state. This contravenes a general principle that suggests that the degree of stereoselectivity should increase with increasing affinity of binding (37). Two possible models may explain this apparent contradiction. In the first, flux is inhibited at a site distinct from that to which pentobarbital binds in the resting state. The former site, which may be in the lumen of the channel, may be too featureless to distinguish the enantiomers, or the

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chiral carbon may not be involved in binding, or two-point binding including the chiral carbon occurs (38). In the second model, pentobarbital binds to the same physical site in all conformational states, with the affinity being highest for the active or open state, intermediate for the resting state, and very much lower for the desensitized state. The second model implies that the chiral carbon of pentobarbital is more intimately involved with the binding site in the resting than in the active state (because stereoselectivity is greater), yet pentobarbital binds with higher affinity to the active state. It is possible that tighter binding of the alkyl chain to the resting state might be offset by unfavorable interactions in the pyrimidine ring region, requiring that the sterochemistry of the binding site must change significantly between the two conformational states. More detailed studies will be required to elucidate these questions.

Although the contrast between the enantiomer's lack of pronounced stereoselectivity as channel blockers and their stereoselective binding at the acetylcholine receptor was unexpected, it should be noted that stereoselective channel inhibition for the acetylcholine receptor is rarely observed (39).

Comparison with stereoselectivity in other systems. Stereoselectivity has been observed in binding of pentobarbital to other receptors. It is of particular interest to compare the stereoselectivity of the action of pentobarbital on the acetylcholine with that on the GABA receptor because, based on the high degree of homology in the transmembrane region of each receptor, it has been proposed that they belong to the same superfamily (40).

Weak stereoselectivity of barbiturate action has been observed at the GABA receptor by several workers. Pentobarbital displaces the cage convulsant TBPT with IC_{50} values of about 130 and 80 μM for the (+)- and (−)-enantiomers, respectively (8, 41). Thus, the stereoselectivity of the barbiturate allosteric site on the GABA receptor is the opposite of that on the acetylcholine receptor, but the (+)-enantiomer has a very similar IC_{50} for both the GABA and acetylcholine receptor whereas the (−)-enantiomer interacts more strongly with the GABA receptor. Pentobarbital also induces chloride currents at the GABA receptor, with the (−)-enantiomer being severalfold more potent than the (+)-enantiomer (42, 43), whereas at higher concentrations pentobarbital causes desensitization of the GABA response with unknown stereoselectivity (44). However, enhancement of GABA currents occurs at higher concentrations than those effective at modulating TBPT binding, and Simmonds and Turner (11) have suggested that enhancement of GABA currents, in spite of its weak stereoselectivity, is a nonspecific effect, related to lipid solubility, whereas the action on TBPT binding involves a more selective site. Certainly weak stereoselectivity in perturbing lipid bilayers has been observed in the case of cannabinoids (45) and opiates (46).

Finally, stereoselective effects of pentobarbital have been observed in more complex systems, such as the crayfish stretch receptor and hippocampal CA1 neurons (2). In the latter case there was a loose parallel with the action of pentobarbital on acetylcholine binding, because in the CA1 neurons pentobarbital exerted a biphasic stereoselective action on the amplitude of the electrically evoked field potentials. At low concentrations (10–100 μM), the amplitude was enhanced with a 5-fold stereoselectivity for the (+)-enantiomer, whereas at higher concentrations (0.1–1 mM) it was depressed with much weaker (1.5-fold) stereoselectivity (2).

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