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A STEREOSELECTIVE PENTOBARBITAL BINDING SITE IN CHOLINERGIC MEMBRANES FROM TORPEDO CALIFORNICA

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Summary: Acetylcholine receptor-rich membranes from Torpedo californica contain a binding site for [^{14}C] pentobarbital which has a dissociation constant of 210 ± 24 μM and 1.4 ± 0.18 sites per acetylcholine site. (+) pentobarbital competes for this site three times more effectively than (-) pentobarbital. Cholinergic ligands decrease [^{14}C] pentobarbital binding and this effect is blocked by pre-incubation with α-bungarotoxin. Pentobarbital decreases [^{3}H] acetylcholine binding non-competitively with an apparent dissociation constant similar to the dissociation constant for [^{14}C] pentobarbital binding. Thus, the pentobarbital and acetylcholine binding sites appear to interact with each other allosterically.

At the neuromuscular junction, the cholinergically elicited cation permeability at the postsynaptic membrane can be non-competitively blocked by many compounds including most classes of local and general anesthetics (For a review see (1)). The availability of acetylcholine receptor-rich membranes from Torpedo has enabled the interactions of these inhibitors with the receptor-ionophore complex to be examined in some detail (Reviewed in (2)). Binding studies have directly demonstrated saturable sites for radiolabelled analogs of histrionicotoxin (3), phencyclidine (4) and a number of local anesthetics (5,6), but not to date for any general anesthetics.

Recent evidence emphasizes the heterogeneity of inhibitor — receptor-ionophore complex interactions. For example, the alcohols act independently of histrionicotoxin (7), there may be two classes of local anesthetic action (8) and actions of general anesthetics on the ionophore do not always parallel those on the cholinergic site (7,9). In contrast to the local anesthetics, the alcohols may act via perturbation of lipids and indeed n-octanol's enhancement of [^{3}H] acetylcholine binding is reversed by pressure (10). However, unlike either the alcohols, the volatile, or most local anesthetics, the bar-

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biturates actually reduce [^{3}H] acetylcholine binding (10). Our preliminary findings, reported here, suggest that this modulation occurs through a stereoselective binding site for pentobarbital located on the acetylcholine receptor-ionophore complex.

METHODS

Acetylcholine receptor-rich membranes were prepared from freshly killed Torpedo californica by differential and sucrose gradient centrifugation as previously described (11). A final velocity gradient step was used (12) to improve the specific activity to 2-3 μmoles of [^{3}H] acetylcholine sites per gram of protein.

[^{14}C] Pentobarbital binding was assayed in Torpedo Ringer (250 mM NaCl, 5 mM KCl, 3 mM $CaCl_2$, 2 mM $MgCl_2$ and 5 mM Na_2PO_4) at pH 7.0. [^{14}C] Pentobarbital was deposited from ethanolic solution in a polypropylene microcentrifuge tube and membrane suspension was added. After equilibration at room temperature (30 minutes), aliquots of this stock were transferred to other tubes and diluted with Torpedo Ringer alone or with added agents. After 30 minutes, 100 μl aliquots were centrifuged for 30 minutes at 135,000 g on a Beckman Airfuge and the supernatant immediately aspirated and saved. The pellet was washed quickly with three 150 μl aliquots of ice cold Ringer and solubilized in 50 μl of 20% sodium dodecylsulfate. Finally, the centrifuge tube was transferred into 6 ml of Liquiscint and counted in a Beckman LS 8000 Scintillation Counter.

The effect of pentobarbital on [^{3}H] acetylcholine binding was determined by ultrafiltration as described previously (10, 11).

All isotopically labelled compounds were from New England Nuclear (Boston, Mass.) The isomers of pentobarbital were a gift from the National Institute of Drug Abuse.

RESULTS

Figure 1 shows results for a single experiment in which part of the membrane bound [^{14}C] pentobarbital was displaced by higher concentrations of unlabelled pentobarbital. Of a total of 425 nanomoles of pentobarbital associated with each gram of membrane protein, 140 nanomoles, or 33 percent, were displaceable by 5 mM pentobarbital. Similar results were obtained over a range of free [^{14}C] pentobarbital concentrations from 0.2 to 10 μM. As expected for this weak acid (pK 8.1) raising the pH up to 9.0 reduced both displaceable and non-displaceable binding in a manner consistent with it being the uncharged form that binds. All other barbiturates tested displaced [^{14}C] pentobarbital binding. The order of increasing potency (approximate IC_{50}, mM) was barbital (10.0), butabarbital (1.0), phenobarbital (1.0) and thiopental (<0.5).

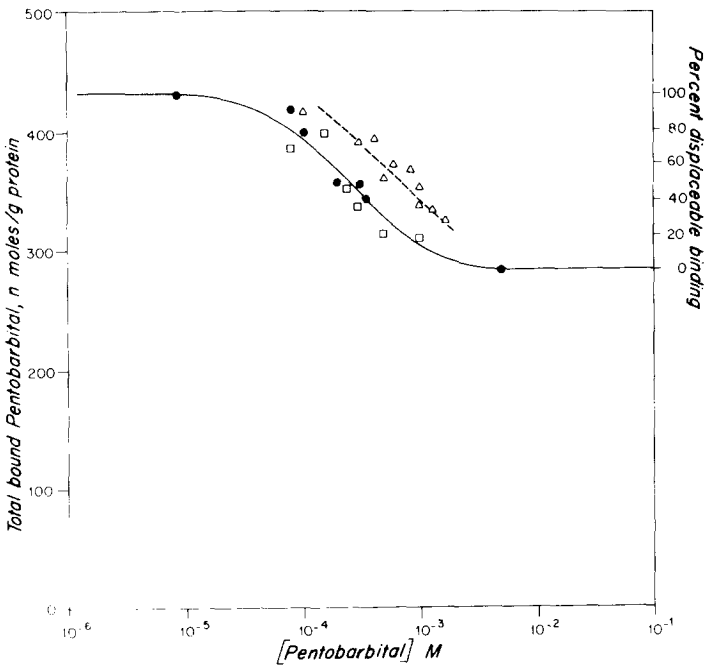

Figure 1. Displaceable binding of [^{14}C](±) pentobarbital. ● (±)pentobarbital; □ (+) pentobarbital; △ (-) pentobarbital. Membranes (0.68 mg protein/ml: 2.88 nmoles acetylcholine sites per mg protein) were incubated with 8.85 µM [^{14}C](±) pentobarbital and the unlabelled pentobarbital indicated. The data for (±) pentobarbital are from a single experiment; the points are the mean of three determinations. The difference between the 8.85 µM and 5.1 mM pentobarbital points was defined as displaceable binding. The points for the optically active pentobarbital isomers are from a separate experiment and have been normalized to the percent displaceable binding scale. The lines were drawn by eye.

On the other hand, the general anesthetic ketamine caused no displacement and urethane only displaced at unphysiologically high concentrations (>125 mM). The amine local anesthetic SKF 525a (proadifen) displaced pentobarbital (20 µM), but in the presence of 50 µM carbachol, which enhances the local anesthetic's affinity for its site about ten fold (5), displacement was achieved at lower SKF 525a concentrations. Although displacement followed mass action in the former case, in the presence of the three ligands this was not so.

In spite of the low proportion of displaceable pentobarbital binding, it proved possible to obtain fair Scatchard plots (Figure 2) which revealed the dissociation constant to be 210 ± 24 µM and the number of pentobarbital sites to be 1.4 ± 0.18 per acetylcholine binding site. The standard deviations given only represent reproducibility. Considering the experimental difficulties,

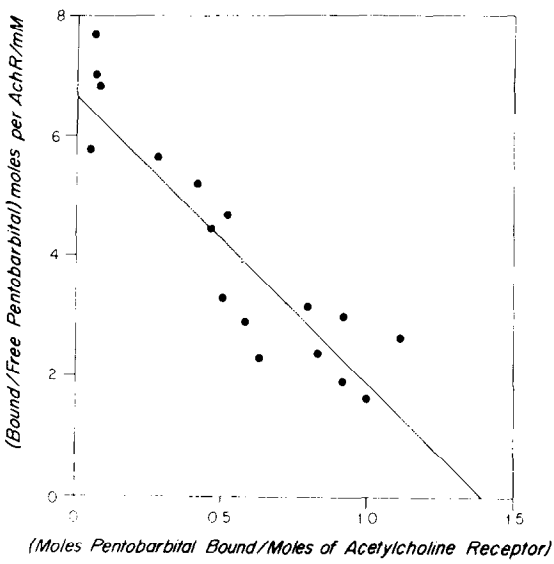

Figure 2. A Scatchard plot of displaceable [^{14}C](+) pentobarbital binding. The points are from four separate experiments and each point represents at least a duplicate determination. Conditions were similar to those in Figure 1. The result of the Scatchard analysis was K_D = 210 ± 24 μM and 1.4 ± 0.18 pentobarbital binding sites per acetylcholine site. The line was fitted by least squares analysis.

we expect non-random errors to be considerably larger. Nonetheless, the rough parity between the number of acetylcholine and pentobarbital sites suggested that the latter site might be on the acetylcholine receptor protein itself. However, block of the acetylcholine binding site by α-bungarotoxin (5 fold excess), or of the acetylcholinesterase site by diisopropyl flurophosphate (10^{-4} M), failed to change the amount of displaceable binding. Nonetheless, both carbachol (50-1,000 μM) and d-tubocurarine (1 mM) reduced displaceable binding by twenty percent. This small effect was reproducible and was absent at 0.1 μM, but present at 3.0 μM carbachol. The suggestion that occupation of the acetylcholine site regulates pentobarbital binding (5) was confirmed in one experiment where 50 and 1,000 μM carbachol reduced the total displaceable binding of 225 nmoles/g by 46 ± 8.8 and 46 ± 15.2 nmoles/g respectively, whereas under similar conditions in membranes pre-incubated with α-bungarotoxin carbachol had no effect.

Conversely, pentobarbital also reduced acetylcholine binding. In a membrane suspension where approximately half the acetylcholine receptors were oc-

cupied with [^{3}H] acetylcholine ([Acetylcholine Receptor] ≈ 15 nM, [Acetylcholine ≈ 15 nM) pentobarbital reduced the ratio ([Bound Acetylcholine]/[Free Acetylcholine]) from 1.0 (control) to a plateau of 0.6 at 1 to 2 mM. (We have previously shown this effect to be caused soley by a reduction in acetylcholine's dissociation constant (10).) The percent of this effect achieved at a given pentobarbital concentration is compared in Figure 3 to the calculated percent occupancy of the pentobarbital site. The effect of pentobarbital on [^{3}H] - acetylcholine binding coincides within experimental error with the expected occupancy of its site. Thus, occupation of the pentobarbital site appears to regulate acetylcholine binding to its site.

A small quantity of the (+) and (-) isomers of pentobarbital was available to us. These showed weak stereoselectivity in displacing [^{14}C] (±) pentobarbital with the (+) isomer having an IC_{50} slightly lower than that of the racemic mixture and the (-) isomer being about three times less potent (Figure 1). The relative ability of these isomers to alter [^{3}H] acetylcholine binding parallels their affinity for the pentobarbital site.

DISCUSSION

Our data demonstrate that there is a saturable binding site for pentobarbital in acetylcholine receptor-rich membranes from *Torpedo californica*. The dissociation constant of pentobarbital for this site is from one to two orders of magnitude higher than any previously reported for radiolabelled ligands in excitable tissues.

Several lines of evidence suggest the barbiturate site is on a protein, probably the receptor-ionophore complex itself. First, the pentobarbital binding site exhibits weak stereoselectivity, although *a priori* this does not rule out stereospecific drug-lipid interactions (13). Second, the ratio of pentobarbital sites to acetylcholine sites is close to unity, suggesting these sites are on the same protein complex. Third, the reduction both of [^{14}C] pentobarbital binding by cholinergic ligands in an α-bungarotoxin sensitive manner, and of [^{3}H] acetylcholine and [^{3}H] d-tubocurarine binding by pento-

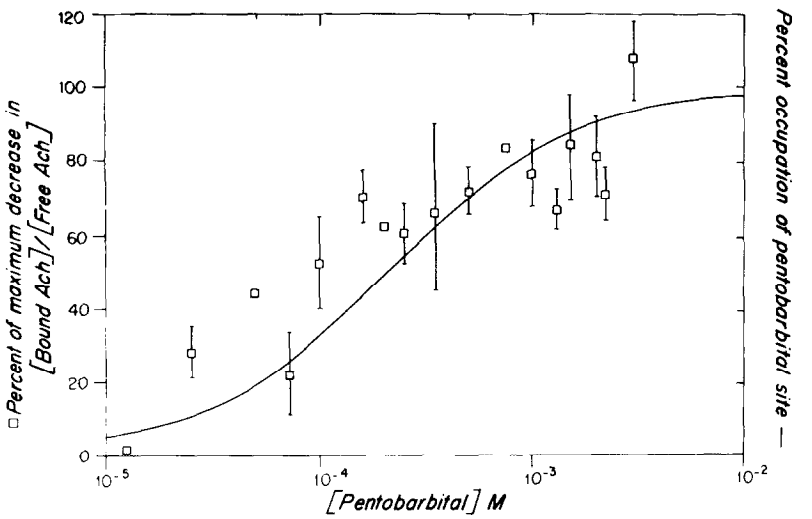

Figure 3. A comparison of pentobarbital's ability to reduce [^{3}H] acetylcholine binding with its ability to occupy its binding site. The line is the calculated percent occupancy of the pentobarbital site using the parameters derived from Figure 2. The squares are the experimentally determined pentobarbital-induced decrease in [^{3}H] acetylcholine binding. This is expressed as a percent of the maximum observed decrease in the ratio [Bound Acetylcholine]/[Free Acetylcholine]. Standard deviations are given where more than one determination was made. The decrease in [^{3}H] acetylcholine binding occurred non-competitively (10).

barbital (10) demonstrates (Figure 3) that the sites are sufficiently coupled to exhibit mutual regulation and are thus probably located on the same protein complex.

All other barbiturates studied to date interact with the pentobarbital site suggesting it acts as a barbiturate receptor. However, it is not a general anesthetic receptor because ketamine does not interact with it and urethane does so only very weakly. We have not yet tested volatile anesthetics for technical reasons. On the other hand, a relationship between the barbiturate site and the previously described local anesthetic site (5) is suggested by the SKF 525a displacement of [^{14}C] pentobarbital binding. Furthermore, the stoichiometry of the two sites is similar (4,14,15) and there is an isolated report that amobarbital displaces [^{14}C] meproadifen binding (7). However, the mutual regulation between cholinergic binding and barbiturate binding is in the opposite direction from that with most aromatic amine binding. This is not incompatible with the sites being identical (7,15), but it does emphasize that the nature of the allosteric interaction between the

two sites may be dependent upon the molecular architecture of the anesthetic. On the other hand, preliminary data suggest that in the presence of carbachol and both pentobarbital and SKF 525a complex interactions may occur.

Although pentobarbital appears to modulate [^{3}H] acetylcholine binding through a site or receptor on the acetylcholine receptor-ionophore complex, this is probably not true of the volatile anesthetics. These enhance [^{3}H] acetylcholine binding over a narrower range of concentration, their effects are independent of the presence of pentobarbital (unpublished data) and are reversed by pressure (10,18). A similar independence of action has been noted between 2-propanol and local anesthetics (7).

Pentobarbital binding also differs from local anesthetic binding by only weakly differentiating between the resting and desensitized conformations of the acetylcholine receptor. However, we have shown pentobarbital to block the carbachol stimulated cation efflux from Torpedo californica membrane vesicles with an apparent dissociation constant of 25 μM (9). One simple explanation for this higher affinity could be that when the receptor-ionophore complex undergoes a transition from the resting to the transient open conformation, the barbiturate site changes conformation in concert such that its affinity for pentobarbital is transiently enhanced by an order of magnitude. Electrophysiological data also support a model where barbiturate binding favors the open state of the receptor-ionophore complex (17). If this is so, then the barbiturate site we have demonstrated would be intimately involved in the function of the cholinergic receptor and its ionophore. Whether this site also has a physiological role, perhaps interacting with endogenous pyrimidines or similar compounds, remains an open question.

ACKNOWLEDGEMENTS

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REFERENCES

(1) Colquhoun, D. (1975) Ann. Rev. Pharmacol. 15, 307-325.
(2) Heidmann, T. and Changeux, J.P. (1978) Ann. Rev. Biochem. 47, 371-411.
(3) Eldefrawi, M.E., Eldefrawi, A.T., Mansour, N.A., Daly, J.W., Witkop, B. and Albuquerque, E.X. (1978) Biochemistry 17, 5474-5483.

(4) Eldefrawi, M.E., Eldefrawi, A.T., Aronstam, R.S., Maleque, M.A., Warnick, J.E. and Albuquerque, E.X. (1980) Proc. Natl. Acad. Sci. 77, 7458-7462.
(5) Krodel, E.K., Beckman, R.A. and Cohen, J.B. (1979) Mol. Pharmacol. 15, 294-312.
(6) Sobel, A., Heidmann, T., Cartaud, J. and Changeux, J.P. (1980) Eur. J. Biochem. 110, 13-33.
(7) Cohen, J.B., Boyd, N.D. and Shera, N.S. (1980) Progress in Anesthesiology, Vol. 2, pp. 165-174, Raven Press, New York.
(8) Heidmann, T. and Changeux, J.P. (1981) FEBS Letters 131, 239-244.
(9) Braswell, L.M., Miller, K.W. and Sauter, J.F. (1981) Brit. J. Pharmacol. 73, 187P.
(10) Sauter, J.F., Braswell, L.M. and Miller, K.W. (1980) Progress in Anesthesiology, Vol. 2, pp. 199-207, Raven Press, New York.
(11) Sauter, J.F., Braswell, L.M., Wankowicz, P. and Miller, K.W. (1981) Underwater Physiology VII, pp. 629-637, Undersea Medical Society, Bethesda.
(12) Jeng, A.Y., St. John, P.A. and Cohen, J.B. (1981) Biochim. Biophys. Acta 646, 411-421.
(13) Loh, H.H., Cho, T.M., Wu, Y.-C. and Way, E.L. (1974) Life Sci. 14, 2231-2245.
(14) Medynski, D.C. and Cohen, J.B. (1981) Soc. for Neurosci. 7, 345.
(15) Elliot, J. and Raftery, M.A. (1979) Biochemistry 18, 1868-1874.
(16) Sauter, J.F., Braswell, L.M. and Miller, K.W. (1979) Pharmacologist 21, 13.
(17) Adams, P.R. (1976) J. Physiol. 260, 531-552.