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CHARACTERIZATION OF [^{3}H]PHENOBARBITAL BINDING TO RAT BRAIN MEMBRANES

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The binding of [^{3}H]phenobarbital to rat brain membranes was studied in order to determine its characteristics and specificity. The binding reaction was rapid and occurred at sites of low affinity. ($K_d = 700$ μM) and very high density ($B_{max} = 2.7$ nmol/mg protein). It was unaffected by temperature changes from 0°C to 95°C and was maximal at pH 5. Detergents in low concentrations markedly decreased the binding, apparently without solubilizing the binding sites. It is concluded that the binding of [^{3}H]phenobarbital is a rather non-specific interaction with the plasma membrane.

The mechanism of action of barbiturates is still unclear [7]. Some authors favor an interaction with plasma membrane lipids [13], which may induce a change in membrane fluidity [4, 5] and consequently an alteration of membrane protein functioning [15]. Others, however, have reported specific interactions of barbiturates with membrane proteins, which may be responsible for the mediation of their anesthetic and anticonvulsant properties [1, 8]. In particular, effects on receptors such as the GABA–benzodiazepine receptor complex [11], the adenosine $A_1(R_i)$ receptor [9] and the thyrotropin-releasing hormone (TRH) receptor [6] have been observed.

In this context radioligand binding studies might be useful in determining the site of action of barbiturates. Using this technique, Willow et al. [17] reported the existence of [^{3}H]phenobarbital binding sites in rat brain membranes and suggested that the study of this binding process might be of use in the investigation of the mechanism of action of barbiturates. Therefore we have studied the binding of [^{3}H]phenobarbital to brain membranes in order to determine its characteristics and possible relevance.

Crude synaptosomal membranes from rat brain were prepared as described by Willow and Johnston [18]. Briefly stated, rat forebrains were homogenized in 10 vols. of 0.32 M sucrose with a glass–Teflon homogenizer and the homogenate centrifuged at 1000 *g* for 10 min. The supernatant was again centrifuged at 14,500 *g* for 20 min. The pellet was resuspended in 20 vols. of 50 mM Tris-citrate buffer, pH

7.1, and centrifuged as above; this washing step was repeated 5 times. Finally the pellet was resuspended in the same buffer at a protein concentration of 10–15 mg/ml, frozen in liquid nitrogen and stored at −80°C. Alternatively, membranes (P_2, B) were prepared according to Whittaker [16]. Protein was determined according to Lowry et al. [10].

The binding assay was carried out in a total volume of 250 μl of 50 mM Tris-citrate, pH 7.1, containing 50 nM [^{3}H]phenobarbital (spec. act. 8.1 Ci/mmol; NEN, Dreieich, F.R.G.) and approximately 300 μg of membrane protein. Other substances or incubation buffers were used as indicated. Incubation was for 20 min at 20°C unless stated otherwise and was terminated by centrifugation at 10,000 *g* for 3 min followed by aspiration of the supernatant. [^{14}C]sucrose (approximately 10,000 cpm/tube) was used to correct for [^{3}H]phenobarbital trapped with incubation buffer in the pellet [12]. The tips of the tubes were cut off and counted in a dual-channel liquid scintillation counter. Non-specific binding was determined in the presence of 30 mM unlabelled phenobarbital and amounted to approximately 30% of total binding; data are given as specific (total minus non-specific) binding. The binding was found to increase linearly with protein concentrations up to 3.5 mg/ml.

The saturation isotherm of [^{3}H]phenobarbital binding and the linear Scatchard plot of the data (Fig. 1A) suggest the presence of a single class of non-interacting binding sites. These binding sites are of low affinity ($K_d = 720$ μM) and extremely high density ($B_{max} = 2.7$ nmol/mg protein). Willow et al. [17] reported an almost ten times higher affinity; however, they also found a similarly high number of binding sites. For this reason it is unlikely that the binding occurred at a specific membrane protein; indeed, if a molecular weight of 50,000 is assumed, these binding sites would have to account for 15% of the membrane protein. Similarly high B_{max} values were found using P_2 and B membrane fractions with no enrichment in the B fraction.

[^{3}H]phenobarbital could be displaced from the binding sites by all barbiturates tested; K_i values (95% confidence limits) obtained from inhibition curves ranged from 0.8 (0.5–1.3) mM for thiopental to 3.1 (2.8–3.4) mM for hexobarbital. Barbituric acid which is pharmacologically inactive has a K_i of 6.5 mM. Thus we could not reproduce the findings by Willow et al. [17] who reported marked differences in the affinity of the barbiturates and almost 100 times lower K_i values for methohexital and thiopental (8 and 11 μM vs 0.8 and 0.9 mM). A variety of other compounds including diazepam, GABA, picrotoxin, ethanol, adenosine and N^6-phenylisopropyladenosine had no effect in concentrations up to 10 mM.

The association and dissociation of the radioligand (Fig. 1B) are complete within 1 min and thus are too fast to be detected by equilibrium binding techniques. This gives further support to the notion of a binding process of low affinity.

In order to test the hypothesis that the radioligand binds to a non-protein site, binding was carried out at different temperatures ranging from 0°C to 95°C (Fig. 1C). Temperature changes, which have pronounced effects on radioligand binding

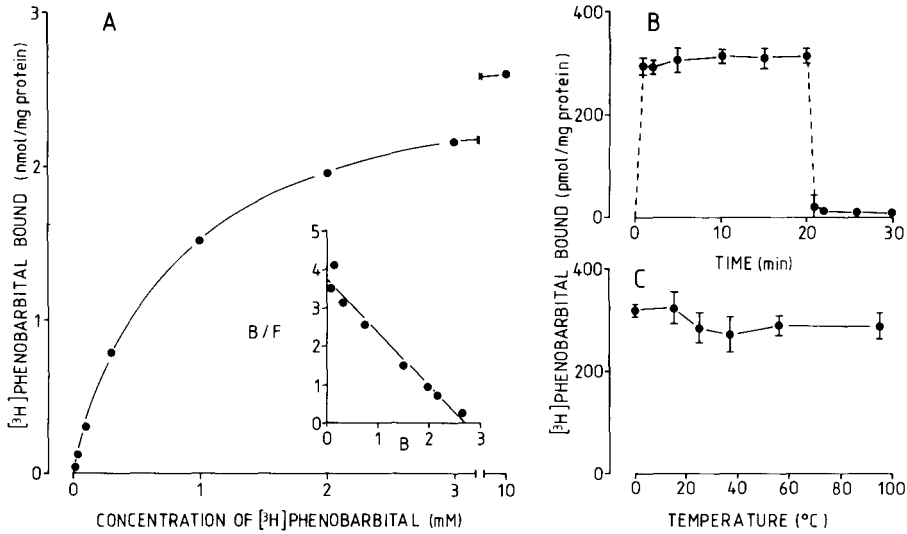

Fig. 1. Characteristics of [^{3}H]phenobarbital binding to rat brain membranes. A: saturation isotherm. The membranes were incubated at 20°C with increasing concentrations of radioligand, which was diluted with unlabelled phenobarbital to obtain high concentrations. The inset shows the Scatchard plot of the data. B, radioligand bound (pmol/mg protein); F, free radioligand (mM). A K_d = value of 721 μM and a B_{max} value of 2.7 nmol/mg protein are obtained. B: kinetics. The association of [^{3}H]phenobarbital (50 nM) was started by addition of membrane protein and the dissociation initiated after 20 min of incubation by addition of 30 mM of unlabelled phenobarbital. Centrifugation time was reduced to 30 s so that total incubation times of 1 min could be obtained. C: effects of temperature. Incubation time was 20 min at all temperatures; this was sufficient to reach equilibrium. Analysis of variance indicates no significant influence of temperature on [^{3}H]phenobarbital binding. Data are the mean and S.E.M. of 3 experiments.

to receptors [2], did not influence the binding of [^{3}H]phenobarbital. Even exposition to 95°C for prolonged periods of time did not appear to alter the binding site. In addition, the binding site was not subject to attack by a variety of proteases, i.e. trypsin, chymotrypsin, pronase, elastase and papain, even in high concentrations (data not shown). The binding was also not affected by concentrations of phospholipase A_2 and C or lipase which cause, for example, marked reduction of binding of calcium channel blockers [3].

Binding of radioligands to receptors usually has an optimum at physiological pH values [2]. In contrast to these findings, binding of [^{3}H]phenobarbital to brain membranes was maximal at pH 5 (Fig. 2A); at lower pH the radioligand is insoluble in aqueous solution. This suggests that the undissociated form which predominates at low pH is preferentially bound by the membranes. This binding of the uncharged molecule might be due to penetration into the lipid bilayer. To test this hypothesis, binding of [^{3}H]phenobarbital was carried out in the presence of increasing concentrations of various detergents (Fig. 2B). Even in concentrations as low as 0.1% all

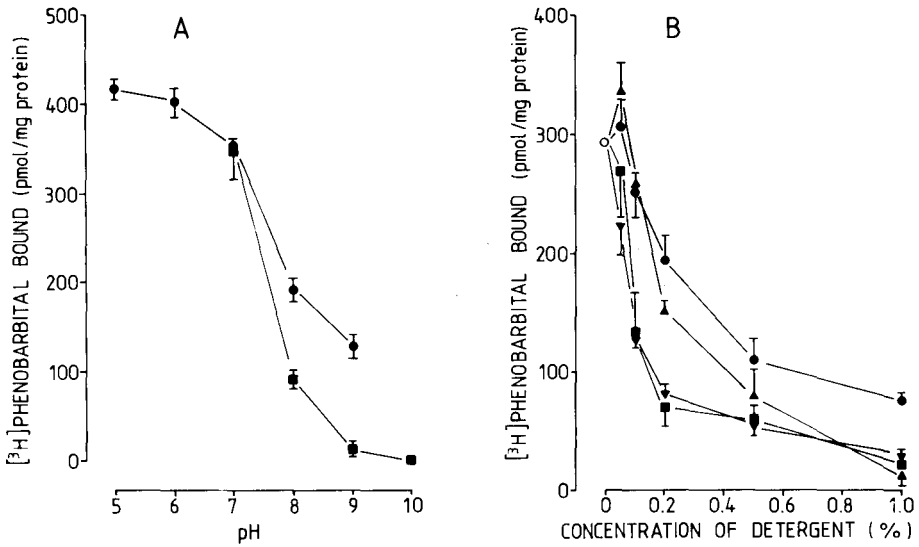

Fig. 2. Effects of pH and detergents on [^{3}H]phenobarbital binding. A: effects of pH. The incubation buffers used were: 50 mM K^+-phosphate buffer (●, pH 5–9) and 50 mM Tris-HCl buffer (■, pH 7–10). B: effects of detergents. Incubation was in the presence of different concentrations of: Na^+-cholate (●), CHAPS [(3-cholamido-propyl)dimethylammonio]propanesulfonate] (▲), Triton X-100 (■) and lubrol PX (▼). All detergents were from Sigma, München, F.R.G. Data are the mean and S.E.M. of 3 experiments.

detergents tested decreased the binding to a considerable extent. At the same concentrations, binding of other radioligands to receptors, i.e. [^{3}H]phenylisopropyladenosine to A_1 adenosine receptors or [^{3}H]yohimbine to α_2-adrenoceptors, in the same tissue was markedly increased (data not shown). To exclude the possibility that the binding sites had been solubilized, binding in the supernatant was tested after the addition of 0.15% γ-globulin and 12% polyethyleneglycol 6000. No binding could be detected in the supernatant obtained with any of the detergents. Therefore the reduction of the binding by detergents appears to be due to an interaction with the lipid bilayer itself.

To summarize our results, the very high number of binding sites and their characteristics suggest that the binding sites for [^{3}H]phenobarbital and the sites mediating barbiturate effects on the GABA-benzodiazepine receptor complex or adenosine A_1 receptor are not identical. This hypothesis is supported by a recent report that barbiturate interactions with the GABA-benzodiazepine receptor complex are retained in a purified receptor preparation [14]. Thus, barbiturates do not seem to regulate these receptors by binding to plasma membrane phospholipids as suggested by Willow et al. [17]. On the contrary it appears that the binding of [^{3}H]phenobarbital to brain membranes is a rather non-specific interaction with the plasma membrane which is also, to a lesser extent, shared by pharmacologically in-

active barbiturates. Although this does not exclude specific interactions of barbiturates with membrane proteins, binding studies with [^{3}H]phenobarbital appear to be of limited usefulness in order to clarify the mechanism of action of barbiturates.

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