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DIFFERENTIAL EFFECTS OF SEDATIVE AND ANTICONVULSANT BARBITURATES ON SPECIFIC [^{3}H]GABA BINDING TO MEMBRANE PREPARATIONS FROM RAT BRAIN CORTEX

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Abstract—The sensitivity to barbiturates of [^{3}H]GABA binding to synaptosomal membrane fractions from rat cortex has been examined. We show that a range of anaesthetic/sedative barbiturates enhance GABA binding in the presence of chloride or other ions that interact with the associated ionophore. Furthermore, picrotoxinin and the anticonvulsant barbiturate phenobarbital antagonise the enhancement produced by pentobarbital. These effects are therefore comparable to those observed at benzodiazepine receptors and may be mediated through the chloride ionophore component of the receptor complex. Other classes of anticonvulsants failed to antagonise pentobarbital activation, suggesting that these interactions may occur at a specific barbiturate site in the membrane.

There is now considerable evidence that pentobarbital and other barbiturates interact with the γ-aminobutyrate (GABA)/benzodiazepine receptor complex which is coupled to a picrotoxinin-sensitive chloride ionophore [1–4]. Some aspects of the physiological actions of the barbiturates may be mediated through this complex [5]. The binding of benzodiazepines to receptor sites in synaptic membrane preparations has been shown to be enhanced by anaesthetic barbiturates in the presence of chloride ions [6, 7] and a recent report [8] has suggested that anticonvulsant barbiturates antagonise this effect.

Although barbiturates have been shown to potentiate the actions of GABA on neurons *in vitro* [1, 2] and *in vivo* [3, 4], efforts to demonstrate the effects of these compounds on Na^+-independent GABA binding have produced widely conflicting results [9–16]. Barbiturates have been variously reported to have no effect on sodium-independent GABA binding [8–11], to increase the affinity of GABA for the high-affinity binding site by slowing its rate of dissociation [12–14] or to increase the apparent number of sites in the membrane [15, 16]. The reported conditions under which these effects may be observed, and their magnitude, vary considerably from one report to another, suggesting that these responses are very sensitive to changes in the method of tissue preparation and assay. We have, therefore, compared the tissue preparations first reported to show barbiturate sensitivity [12, 15] in order to attempt to define conditions under which the effects of barbiturates may be reproducibly demonstrated.

MATERIALS AND METHODS

4-Amino-*n*-[2,3-^{3}H]butyric acid (64 Ci/mmole) was purchased from the Radiochemical Centre (Amersham, U.K.). GABA, pentobarbital, secobarbital, hexobarbital, bicuculline, picrotoxinin and nipecotic acid were obtained from Sigma (London) Chemical Co. (Poole, U.K.) and diphenylhydantoin was from Aldrich Chemical Co. (Gillingham, U.K.). Ethosuccimide, sodium valproate and carbamazepine were gifts from Dr D. S. Walter, Reckitt & Colman Pharmaceutical Division (Hull, U.K.). All other chemicals were obtained from British Drug Houses Ltd. (Poole, U.K.).

Preparation of lysed synaptosomal membranes. Two different methods of membrane preparation were investigated. The first procedure was that of Willow and Johnston [12]. Brains were obtained from male Wistar rats (150–200 g) and cortices rapidly removed and homogenised in 10 vol. ice-cold 0.32 M sucrose, pH 7.5. After an initial centrifugation at 1000 *g* for 10 min, the supernatant was centrifuged at 20,000 *g* for 20 min to obtain a crude synaptosomal pellet (P_2). This pellet was then washed 10 times with ice-cold 50 mM Tris–citrate buffer, pH 7.1, by resuspension and centrifugation.

The alternative preparation, as described by Olsen *et al.* [15] involved the washing of the crude synaptosomal pellet (P_2) twice by resuspension in ice-cold distilled water, followed by centrifugation at 48,000 *g* for 20 min. The pellet was then washed once with ice-cold 20 mM potassium phosphate buffer, pH 7.5, containing 50 mM KCl, resuspended in a minimum volume of the same buffer and dialysed against 100 vol. of buffer for 20 hr prior to receptor binding assay.

Protein was determined by the method of Lowry *et al.* [17].

GABA receptor binding assay. The binding of [^{3}H]GABA to synaptosomal membranes was performed by a centrifugation assay. Protein (0.6–0.8 mg) was incubated for 5 min at 2° in the presence of 4 nM [^{3}H]GABA and various concentrations of drugs, in a total vol. of 1 ml. Non-specific binding

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was measured in the presence of 1 mM GABA and was unaffected by any of the drugs used. Samples were then centrifuged (48,000 g, 10 min) and the pellets washed twice with ice-cold buffer, solubilised in NCS Tissue solubiliser and counted in 5 ml scintillation fluid (0.5% PPO in toluene). All experimental points were performed in duplicate. Under these assay conditions basal, specific [^{3}H]GABA binding (measured in the absence of drugs) was totally abolished by bicuculline, but was unaffected by 1 mM nipecotic acid, an inhibitor of GABA uptake.

RESULTS AND DISCUSSION

Sodium-independent binding of GABA to synaptic membranes has characteristics consistent with the labelling of post-synaptic receptors. Fresh, undialysed membrane preparations show a single class of sites, whereas freeze/thawed and thoroughly washed membranes, or membranes pre-treated with low concentrations of Triton X-100 reveal a second class of 'high affinity' sites. This change in binding characteristics may be attributed to the removal of endogenous inhibitors, in particular endogenous GABA [18, 19]. It is therefore important that preparations used to measure GABA binding should be free of such inhibitors.

For initial binding studies we used the washed membrane preparation described by Willow and Johnston [12]. However, even after 10 washes, the supernatant still retained the ability to displace [^{3}H]GABA from the membrane preparation to a significant extent, suggesting that the washing procedure failed to remove all endogenous inhibitors (Table 1). Furthermore, [^{3}H]GABA binding in this preparation was considerably lower (approx. 50%) than in membranes prepared by osmotic shock followed by a single wash with 50 mM Tris–citrate buffer, pH 7.1. This may be due to a disruption of the integrity of the receptor complex during the lengthy, manipulative procedure. No effect of pentobarbital was observed in the range of 10–200 μM.

Success in observing barbiturate-sensitive [^{3}H]GABA binding was achieved using membranes prepared by osmotic shock followed by dialysis and assay in 20 mM potassium phosphate buffer, pH 7.5, containing 50 mM KCl. This method yields membranes with higher specific [^{3}H]GABA binding compared to undialysed preparations [20]. Prior to dialysis, these membranes showed a single binding site for GABA with a K_D value (220 ± 80 nM) similar to that obtained by ourselves and others (e.g. Refs 18, 20, 21) for fresh membranes prepared and assayed in Tris–citrate buffer pH 7.1. After dialysis, however, Scatchard analysis revealed two binding sites with affinities similar to those obtained from frozen/thawed or detergent-treated membranes [10, 19, 21–23] (K_{D_1} = 15 ± 5 nM, K_{D_2} = 150 ± 50 nM), suggesting that this preparation was free from endogenous inhibitors. Dialysis, therefore, appears to provide a less disruptive and more efficient method for removal of endogenous inhibitors than the conventional procedure of repeated washing. A range of barbiturates was shown to enhance the specific binding in this preparation at concentrations above 10 μM (Fig. 1). Maximal enhancement varied from 57% to zero depending on the barbiturate tested, with secobarbital being the most potent and phenobarbital the least. These results show a close correlation with those obtained for the effects of barbiturates on benzodiazepine binding [7]. In all cases, the activation appeared to be saturable. In contrast to the recent work of Willow and Johnston [24], we did not detect a reversal of activation by concentrations of pentobarbital greater than 0.5 mM. A small reduction in activation by barbital was observed at concentrations above 25 mM, but this would seem unlikely to have any physiological significance. Scatchard analysis in the presence of pentobarbital suggests that the drug increases the apparent total number of sites (B_t) in the membrane

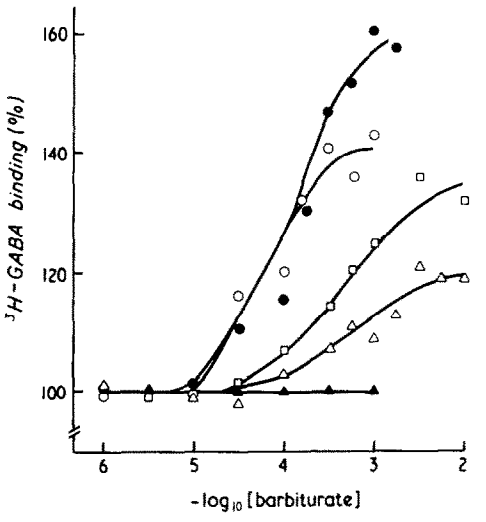

Fig. 1. Effects of a range of barbiturates on [^{3}H]GABA binding to fresh rat brain cortex membranes. Receptor binding assays were performed as described in Materials and Methods, in 20 mM potassium phosphate buffer, pH 7.5, containing 50 mM KCl, using 4 nM [^{3}H]GABA. Results are the mean of four separate experiments. (▲) phenobarbital, (△) barbital, (□) hexobarbital, (○) pentobarbital, (●) secobarbital.

Table 1. Inhibition of [^{3}H]GABA binding by supernatant fractions from repeated washing of synaptosomal membranes

No. of washes	% Inhibition of GABA binding
1	92.9 ± 3.7
3	67.1 ± 2.0
5	53.3 ± 2.9
7	49.4 ± 1.5
9	45.4 ± 4.1
10	39.1 ± 5.1

The binding of [^{3}H]GABA (4 nM) to synaptosomal membrane fractions was measured in the presence of supernatant (500 μl) derived from each of 10 consecutive washes of the membranes with ice-cold 50 mM Tris/citrate buffer, pH 7.1 (see Materials and Methods). The results represent the mean ± S.E.M. of 3 separate determinations.

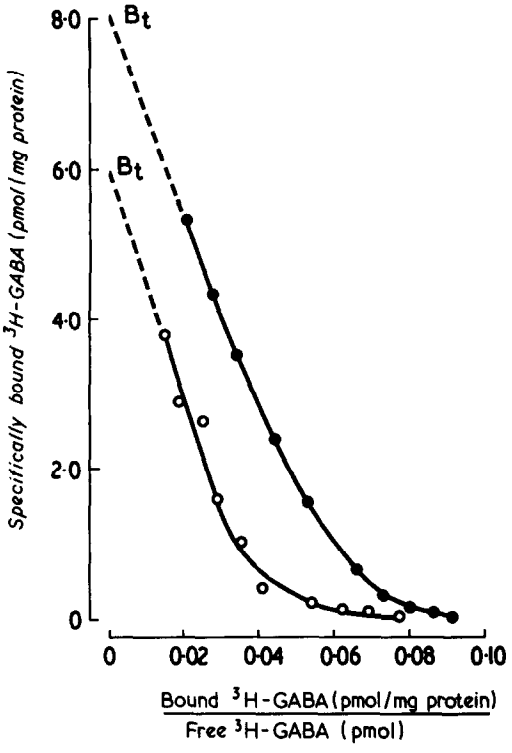

Fig. 2. Scatchard analysis of [³H]GABA binding in the absence (○) and presence (●) of 0.5 mM pentobarbital. The concentration of GABA was varied in the range 0.4–254 nM. Results are typical of three separate experiments.

(Fig. 2). This is in agreement with the work of Olsen *et al.* [15] and Asano and Ogasawara [16], but in contrast to that of Willow and Johnston [12, 14] who reported a change in the affinity of the 'high affinity' binding site, but no change in the total number of sites. The activation by barbiturates was inhibited in a dose-dependent manner by picrotoxinin, with abolition of enhancement at 10 μM. In agreement with other authors [25, 26] we were unable to observe any effect of this toxin on basal levels of [³H]GABA binding.

The anticonvulsant barbiturate phenobarbital had no direct effect on the binding of [³H]GABA to rat brain cortex even at a concentration of 1 mM (Fig. 1). In the presence of 0.5 mM pentobarbital, however, phenobarbital was shown to abolish the activation induced by the anaesthetic barbiturate (Fig. 3). Activation was reduced to 50% of maximum at 240 μM phenobarbital, and a return to pre-activation level was achieved at 1 mM. This reversal is unlikely to be due to the increased total concentration of barbiturate as suggested by Willow and Johnston [24], since an effect is detectable at concentrations that do not increase the total barbiturate concentration to more than 1 mM. At this concentration, no reversal is seen with pentobarbital alone. In addition, other barbiturates, e.g. barbital (Fig. 3) and hexobarbital (not shown) have no effect on pentobarbital-induced activation over a similar concentration range. Thus this effect appears to represent a true antagonism of pentobarbital enhancement by phenobarbital.

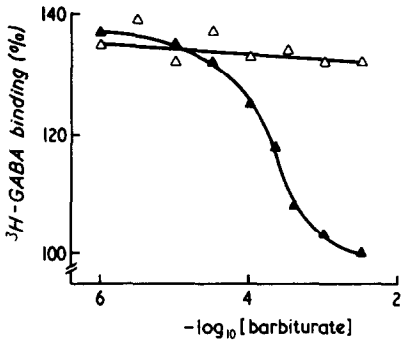

Fig. 3. The effect of phenobarbital (▲) on [³H]GABA binding in the presence of 0.5 mM pentobarbital. Barbital (△) is included for comparison. Each set of data represents mean values of four separate determinations.

Sensitivity to activation by pentobarbital was dependent on the presence of chloride or other ions, e.g. I^-, Br^-, that have been shown to permeate the GABA-regulated anion channels involved in inhibitory synapses in the spinal cord [27]. Other anions, including sulphate, acetate, phosphate and citrate, were inactive at producing enhancement of GABA binding by pentobarbital, confirming that this effect is not due simply to an increase in ionic strength. Furthermore, there is no involvement of the cation in this process, since ammonium chloride was equally as effective as potassium chloride.

No pentobarbital-induced activation was demonstrable in the absence of chloride with either fresh membranes or membranes frozen at −20° for 24 hr prior to dialysis. The effect of chloride ions is concentration-dependent and saturable, with maximum activation occurring at 150 mM. Fifty per cent activation was obtained at 28 mM in fresh membranes and at 37 mM in frozen membranes (Fig. 4).

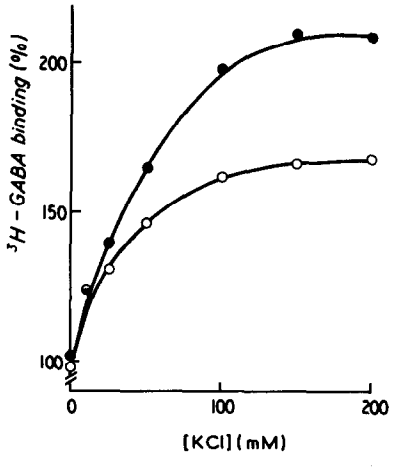

Fig. 4. The effect of chloride concentration on pentobarbital-induced enhancement in fresh (○) and frozen (●) membrane preparations. Pentobarbital concentration was fixed at 0.5 mM and the concentration of KCl varied in the range 0–200 mM. Results are the mean of four separate determinations.

The effect of 0.5 mM pentobarbital was greater in frozen tissue, producing a maximal enhancement of 210%, compared to 167% in fresh tissue. The lack of any detectable activation by pentobarbital in the absence of chloride is in contrast to the results of Willow and Johnston [12] and Asano and Ogasawara [16], but in agreement with those of Olsen *et al.* [15] who were able to demonstrate barbiturate sensitivity in chloride-containing media, but not in Tris–citrate buffer [28]. The difference is not due to the change in buffer from Tris–citrate to potassium phosphate since it was possible to elicit barbiturate sensitivity in membranes prepared and assayed in Tris–citrate by the addition of chloride ions. The results obtained are indistinguishable from those observed in potassium phosphate buffer.

These results show a striking similarity to those obtained for the effects of barbiturates at benzodiazepine receptor sites in terms of chloride-dependence [7] as well as in the antagonistic effects of picrotoxinin [7] and phenobarbital [8]. Thus, the effects of barbiturates may be mediated allosterically via the chloride ionophore. The anticonvulsant valproate (2-propylpentanoate: Epilim®) also appears to have a post-synaptic mode of action [29, 30] and has recently been reported to interact with the ionophore component of the receptor complex [31]. However, in the present work, valproate was unable to reverse the activation induced by 0.5 mM pentobarbital even at 5 mM. Valproate also did not affect basal levels of GABA binding in the absence of pentobarbital. Other classes of anticonvulsant drugs tested, including diphenylhydantoin, carbamazepine and ethosuccimide, also failed to affect [^{3}H]GABA binding in the presence or absence of 0.5 mM pentobarbital. This suggests that the site of action may be barbiturate-specific rather than anticonvulsant-specific. It has been suggested [5, 32] that barbiturates may have two independent modes of action on GABA neurotransmission, related to their sedative and anticonvulsant properties. The potentiation of GABA binding observed in this study appears to relate to the sedative, rather than the anticonvulsant effects. This hypothesis is further substantiated by the relative potencies of the barbiturates tested (secobarbital > pentobarbital > hexobarbital > barbital > phenobarbital) which shows good correlation with the relative anaesthetic potencies and relative activities of these drugs to reverse the antagonism of GABA responses by bicuculline [2]. Further studies are required in order to assess whether the effect of phenobarbital observed in this work is related to the anticonvulsant properties of this compound.

Although the binding of both [^{3}H]GABA and [^{3}H]diazepam is enhanced by pentobarbital, the mechanism of the observed activation appears to be different. Enhancement of benzodiazepine binding is due to an increased affinity of the receptor for its ligand [7] whereas the effect on [^{3}H]GABA binding appears to be due to an increase in the number of detectable sites (Fig. 2). One explanation for this effect [33] is that pentobarbital reveals a class of GABA receptors that were previously undetectable. This may well represent the novel, low-affinity receptor [34] that has been proposed to mediate the effects of GABA on benzodiazepine receptor sites. The concentration of GABA required to enhance [^{3}H]diazepam binding (50% activation at 1.6 μM GABA) [35] is much greater than would be expected from the K_D values reported for the two classes of GABA receptors normally measured. This hypothesis is further supported by the distinct effects of detergents and sulphydryl reagents on GABA binding and on the stimulation by GABA of benzodiazepine binding in rat brain [36]. This low-affinity class of GABA receptors may mediate some aspects of barbiturate action *in vivo*, via the chloride ionophore. Analysis of the effects of other groups of drugs on this complex are currently in progress.

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