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Characteristics of $GABA_B$ receptor binding sites on rat whole brain synaptic membranes

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1 Saturable binding of (±)-[^{3}H]-baclofen and [^{3}H]-γ-aminobutyric acid ([^{3}H]-GABA) to rat brain crude synaptic membranes has been examined by means of a centrifugation assay.

2 The binding of [^{3}H]-baclofen could be detected in fresh or previously frozen tissue and was dependent on the presence of physiological concentrations of Ca^{2+} or Mg^{2+} although a lower affinity Na^+-dependent component could also be observed. Both components probably reflect binding to receptor recognition sites.

3 The saturable portion of bound [^{3}H]-baclofen formed 20.3 ± 6.9% of total bound ligand. This could be displaced by GABA (IC_{50} = 0.04 μM), (−)-baclofen (0.04 μM) and to a much lesser extent by (+)-baclofen (33 μM). Isoguvacine, piperidine-4-sulphonic acid and bicuculline methobromide were inactive (up to 100 μM) and muscimol was only weakly active (IC_{50} = 12.3 μM).

4 Saturable binding of [^{3}H]-GABA increased on adding $CaCl_2$ or $MgSO_4$ (up to 2.5 mM and 5.0 mM respectively) to the Tris-HCl incubation solution. This binding ($GABA_B$ site binding) was additional to the bicuculline-sensitive binding of GABA ($GABA_A$ site binding) and could be completely displaced by (−)-baclofen (IC_{50} = 0.13 μM).

5 Increasing the Ca^{2+} concentration (0 to 2.5 mM) increased the binding capacity of the membranes without changing their affinity for the ligand.

6 The binding of [^{3}H]-GABA to $GABA_B$ sites could be demonstrated in fresh as well as previously frozen membranes with a doubling of the affinity being produced by freezing. Further incubation with the non-ionic detergent Triton-X-100 (0.05% v/v) reduced the binding capacity by 50%.

7 The pharmacological profile of displacers of [^{3}H]-GABA from $GABA_B$ sites correlated well with that for [^{3}H]-baclofen displacement. A correlation with data previously obtained in isolated preparations of rat atria and mouse vas deferens was also apparent.

8 It is concluded that [^{3}H]-baclofen or [^{3}H]-GABA are both ligands for the same bicuculline-insensitive, divalent cation-dependent binding sites in the rat brain.

Introduction

Receptors for the amino acid neurotransmitter γ-aminobutyric acid (GABA) are clearly not homogeneous. For example, the pharmacological characteristics of GABA receptors associated with pre- and postsynaptic Cl^- conductance mechanisms may differ from those mediating enhancement of the binding of benzodiazepines to their receptors (Tallman, Thomas & Gallager, 1978; Karobath, Placheta, Lippitsch & Krogsgaard-Larsen, 1979; Braestrup, Nielsen, Krogsgaard-Larsen & Falch, 1979). They may also differ from autoreceptors thought to be present within the mammalian brain (Mitchell & Martin, 1978; Snodgrass, 1978; Brennan & Cantrill, 1979). Nistri & Constanti (1979) have even suggested that postsynaptic receptors associated with Cl^- channels may be heterogeneous. They believe that spinal interneurones and motoneurones have a predominant receptor type which is distinct from that which predominates on cortical neurones.

An additional separation ($GABA_1$ and $GABA_2$) has been proposed on the basis of different binding affinities for [^{3}H]-GABA exhibited by rat brain synaptic membranes (Guidotti, Gale, Suria & Toffano, 1979; Costa, 1981). However, the pharmacological separation of these and the preceding receptor subtypes is not always immediately apparent. In particular, they can all be blocked by the

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GABA antagonist, bicuculline (Curtis, Duggan, Felix & Johnston, 1970).

We have recently described the existence of another receptor for GABA which shows marked differences from the previously mentioned subtypes (Bowery, Doble, Hill, Hudson, Shaw, Turnbull & Warrington, 1981). Notably the majority of accepted GABA mimetics are not agonists for the receptor and all of the reported GABA antagonists including bicuculline are not active at this site. The GABA derivative, baclofen (β-*p*-chlorophenyl GABA), is a stereospecifically active agonist at the receptor, in striking contrast to its inactivity at conventional GABA receptors (Curtis, Game, Johnston & McCulloch, 1974; Davies & Watkins, 1974; Olpe, Koella, Wolf & Haas, 1977; Ault & Evans, 1978; Bowery *et al.*, 1981). The baclofen-sensitive receptor is present on peripheral autonomic nerve terminals (Bowery *et al.*, 1981) and within the mammalian CNS, where it can influence radiolabelled neurotransmitter output from brain slices (Bowery, Hill, Hudson, Doble, Middlemiss, Shaw & Turnbull, 1980b).

The presence of this receptor in the CNS can also be detected readily by use of a radiolabelled binding technique with [^{3}H]-baclofen or [^{3}H]-GABA as ligands (Hill & Bowery, 1981). We now describe more fully the properties of this binding site which we have designated as the $GABA_B$ receptor, in contrast to bicuculline-sensitive receptors which we have collectively designated $GABA_A$ sites (Hill & Bowery, 1981).

Methods

Tissue preparation

Crude synaptic membranes were prepared from whole rat brain according to the method of Gray & Whittaker (1962) as modified by Zukin, Young & Snyder (1974). Male Wistar rats were killed by decapitation. The brains were removed and homogenized immediately in 15 volumes ice-cold 0.32 M sucrose using a Potter-Elvehjem homogenizer fitted with a teflon pestle (radial clearance 0.25 mm). The homogenate was centrifuged at 1000 *g* to give a crude nuclear pellet (P_1) and a supernatant fraction enriched in myelin, synaptosomes and mitochondria. The supernatant was collected and recentrifuged for 20 min at 20,000 *g*. The resulting pellet (P_2) was lysed by resuspension in distilled water and the mixture centrifuged for 20 min at 8,000 *g*. The supernatant was used to rinse off the upper layer of the pellet with the aid of a Pasteur pipette. The combined suspension was centrifuged for 20 min at 48,000 *g*. The final crude synaptic membranes were washed twice with distilled water and were either resuspended in buffer for further washing prior to use as fresh tissue or were stored frozen at − 20°C.

Triton-X-100 treatment

To investigate the effect of the non-ionic detergent Triton-X-100, previously frozen membranes were thawed for 20 min, resuspended in the appropriate buffer and incubated at 20°C for 20 min. After centrifugation at 7,500 *g* for 10 min the tissue was resuspended in fresh buffer containing 0.05% v/v Triton-X-100 detergent, and maintained at 35°C for 30 min. The detergent-treated tissue was centrifuged at 48,000 *g* for 20 min and the final pellet washed twice in fresh buffer by resuspension and centrifugation at 48,000 *g* for 20 min, before resuspension for assay.

Radioligand binding assay

Throughout the present study, all synaptic membranes which had not been treated with Triton-X-100 were subjected to an extensive washing procedure to remove endogenous GABA and other possible inhibitory substances. Freshly prepared or frozen and thawed membranes were resuspended in the appropriate buffer (either Krebs-Henseleit solution, 50 mM Tris-citrate pH 7.1 or 50 mM Tris-HCl pH 7.4 containing one or more ionic species) and incubated under ambient conditions for 45 min before centrifugation at 7,500 *g* for 10 min. The tissue was washed a further 3 times by resuspension and recentrifugation with incubation periods of 15 min between spins. The final tissue was resuspended in fresh buffer for assay.

In certain other experiments the effect of variations in the concentration of different ionic species on the binding to the $GABA_B$ site was investigated. This necessitated a slightly modified washing procedure which was performed as follows. Frozen membranes were thawed and resuspended in 50 mM Tris-HCl buffer pH 7.4 and incubated at room temperature for 45 min. The suspension was centrifuged for 10 min at 7,500 *g* and the pellet resuspended in fresh Tris-HCl buffer before being divided into aliquots in micro test tubes. After incubation for 15 min the membranes were pelleted and then resuspended in Tris-HCl pH 7.4 containing the appropriate concentration of ion, incubated for 15 min and centrifuged. This procedure was repeated once more before the membranes were finally resuspended for assay.

For the binding assay, membranes were either resuspended in buffer solution and transferred in 0.8 ml aliquots to microtubes (0.5 − 1.0mg protein/tube) or, where the membranes were already present in the tubes, 0.8 ml of buffer solution was added to the pellet for resuspension.

[^{3}H]-GABA (10 nM final concentration:

65 Ci/mmol) or [^{3}H]-baclofen (20 nM final concentration: 8.8 Ci/mmol) was added to each tube with or without varying concentrations of unlabelled test compound (final volume 1.0 ml). All drugs were made up using either buffer solution or distilled water for subsequent dilution into incubation solution. Where additional compounds were required to be present throughout the incubation, these were added to the buffer solution containing the membranes prior to pipetting into aliquots. The final mixture was incubated under the required conditions (10 min at room temperature unless otherwise stated) and the assay was terminated by centrifugation at 7,500 *g* for 10 min. Where incubations were performed at temperatures other than ambient, the tubes containing the membranes were pre-incubated in a water bath for 5 min before addition of any drug. Following centrifugation the supernatant was aspirated off and any remaining fluid blotted from the surface of the pellet with paper tissue. Superficial rinsing of the pellet was not carried out.

The tissue was digested using 'Soluene-350' tissue solubilizer at 35°C and the test tube and its contents were transferred to a scintillation vial containing 400 µl of 0.2 M HCl to neutralize the 'Soluene'. The tritium content of each sample was estimated by liquid scintillation spectrometry.

Uptake studies

Uptake of [^{3}H]-GABA and [^{3}H]-baclofen by rat brain slices was measured using the method of Iversen & Neal (1968). Male rats were killed and the brains rapidly dissected out. The cerebral cortex was flattened with a spatula and the slab of tissue was cut at 0.1 mm intervals with a McIlwain chopper in two directions at 90°. The resulting prisms of brain tissue were resuspended by means of a Pasteur pipette in 2–4 ml of Krebs-Henseleit solution at a final tissue concentration of 10 mg/ml. Aliquots of suspension were distributed volumetrically into conical flasks containing [^{3}H]-GABA (6 nM final concentration: 65 Ci/mmol) or [^{3}H]-baclofen (20–30 nM: 8.8 Ci/mmol) with or without an excess (200 µM) of the same unlabelled drug. Quadruplicate determinations were made for each combination.

Slices were incubated in a shaking water bath at 25°C for the required period of time (between 2 and 45 min). At the end of the incubation the tissue was recovered by rapid filtration on a small Buchner funnel fitted with a Whatman No. 1 filter disc (2.5 cm diameter). The filter discs together with the tissue were placed in scintillation vials and the radioactivity was extracted into 1 ml of water by incubation at room temperature for 2–3 h. Radioactivity was measured by liquid scintillation spectrometry.

Protein determinations

Estimates of the protein content of tissues were made by the method of Lowry, Rosebrough, Farr & Randall (1951) with bovine serum albumin as a standard.

Analysis of results

Non-specific binding was estimated in the presence (usually 100 µM) of non-radioactive drug. This value was subtracted from the total amount of radioactivity bound in the absence of any drug to yield the amount of ligand specifically bound. The specific portion of bound radioactivity was then expressed either as a function of protein concentration or as a percentage of the total radioactivity bound. Alternatively, the amount of radioactivity specifically bound at a given concentration of non-radioactive drug was expressed as a percentage of the total ligand specifically bound.

Saturation experiments were performed with a fixed concentration of radioligand, the specific activity of which was altered by increasing the concentration of non-radioactive compound. After estimation of the amount of radioactivity specifically bound at a given concentration of non-radioactive drug, this value was then used to determine the total amount of ligand bound, according to the formula:

$$B = b \times (1 + \frac{C}{H})$$

where B = Total ligand specifically bound (pmol/mg protein); b = radioactive ligand specifically bound (pmol/mg protein); C = molar concentration of non-radioactive ligand; H = molar concentration of radioactive ligand.

These data were then used for saturation analysis by the method of Scatchard (1949).

In cases where curvilinear Scatchard plots were obtained the binding data were also analysed by hyperbolic plot using computer assisted iterative methods after appropriate weighting (either by standard deviation or the method of Ottaway, 1973) of the data. The most appropriate model was fitted to the data and the binding constants thus estimated were used to construct the individual components of the Scatchard plot. All computer analyses were performed by Dr Ralph White of the Wellcome Research Laboratories.

Materials

The following were used: ^{3}H-[2,3]-4-aminobutyric acid (65 Ci/mmol) (RadioAmersham Ltd); [^{3}H]-baclofen (β-(4-chloro-3-^{3}H(N)phenyl γ-aminobutyric acid) (8.8 Ci/mmol) (Ciba- Geigy Ltd); baclofen and its isomers (Ciba-Geigy Ltd, unless

specified, baclofen indicates racemic form); tris-Aristar (BDH); baclofen analogues (Dr P.J. Roberts, Southampton); GABA (BDH); β-hydroxy GABA (Sigma Ltd); 3-aminopropane sulphonic acid (3-APS) (Sigma Ltd); piperidine-4-sulphonic acid) (P4S), THIP, isoguvacine (Dr P. Krogsgaard-Larsen, Copenhagen); muscimol (Dr M.J. Turnbull, ICI, Alderley Park); δ-aminovaleric acid (Sigma Ltd); picrotoxin, (±)-nipecotic acid hydrochloride, (+)- and (−)-bicuculline methobromide, β-chloro GABA (Dr J.F. Collins, London).

Results

[^{3}H]-baclofen binding sites

Preliminary experiments with Tris-citrate buffer solution (50 mM, pH 7.1), the medium frequently employed in [^{3}H]-GABA binding assays (e.g. Zukin *et al.*, 1974; Enna & Snyder, 1977), indicated that this incubation solution was unsuitable for the detection of saturable binding sites for [^{3}H]-baclofen. The amount of tritium bound to crude synaptic membranes after incubation with [^{3}H]-baclofen (5–20 nM) at 20°C was the same in the absence or presence of a high concentration of unlabelled (±)-baclofen (100 μM). This was true for all incubation times between 5 and 60 min.

Since in our earlier experiments with more intact isolated tissues we had employed Krebs-Henseleit solution (KHS) to detect $GABA_B$ sites (Bowery *et al.*, 1981) we wondered whether the same solution could be used for radioligand binding studies. Arguably the presence of Na^+ or other ions might facilitate binding of [^{3}H]-baclofen to transport recognition sites. Thus any saturable binding detected in KHS could result from an intracellular accumulation of the ligand in a manner analogous to that of GABA. As a preliminary therefore, slices of rat cerebral cortex were incubated at 25°C with [^{3}H]-baclofen (20 nM) using the technique of Iversen & Neal (1968). Tissue:medium ratios obtained after incubation for periods up to 45 min were always less than unity and there was no change in the values when 200 μM unlabelled (±)-baclofen was also present. Conversely in the same experiments after only 10 min incubation with [^{3}H]-GABA (6 nM) a mean tissue:medium ratio of 34:1 was obtained. This was reduced to 1.3:1 in the presence of 200 μM unlabelled GABA. The use of KHS therefore seemed justified since any saturable component observed with [^{3}H]-baclofen would not result from binding to a cellular membrane transport system.

In the presence of KHS, saturable binding of [^{3}H]-baclofen (5–20 nM) to frozen and thawed crude synaptic membranes could be readily detected as

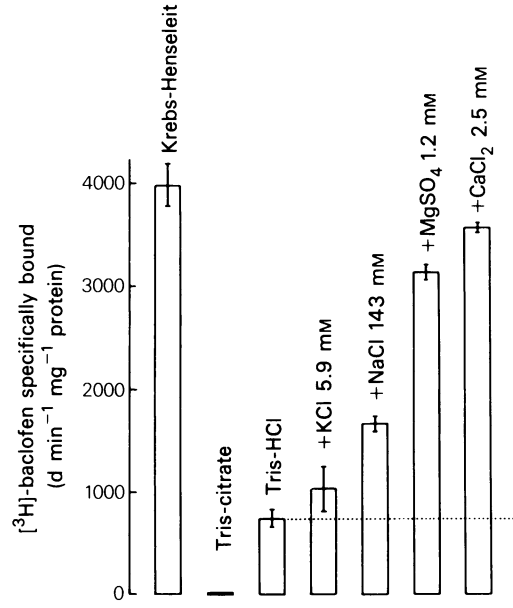


Figure 1 The effect of different incubation media on the saturable component of [^{3}H]-baclofen binding. Frozen and thawed membranes were washed four times in the appropriate medium (indicated above histogram bars) before resuspension for assay at 18°C for 10 min in the same fresh medium. [^{3}H]-baclofen 20 nM was used throughout and non-specific binding which represented approximately 12,000 d min^{-1} mg^{-1} protein irrespective of the incubation medium was determined by (±)-baclofen 100 μM. Each histogram bar represents the mean amount of [^{3}H]-baclofen specifically bound/mg protein (± s.e.mean, $n = 3-9$ experiments in triplicate). The dotted line represents the basal level of specific binding detected in Tris-HCl alone without any added ions. No specific binding of [^{3}H]-baclofen could be detected in Tris-citrate (50 mM, pH 7.4) solution. The apparent difference between the amounts of tritium specifically bound in Tris-HCl alone and Tris-HCl plus KCl was not significant (unpaired t test) whereas the addition of NaCl to Tris-HCl did produce a small and significant increase in binding. The presence of either $CaCl_2$ or $MgSO_4$ produced a large increase in the amount of specifically bound tritium.

illustrated in Figure 1 (column 1). The saturable component determined with 100 μM (±)-baclofen formed 20.3 ± 6.9% (mean ± s.e.: $n = 20$, in triplicate) of total ligand bound. Similar results were obtained with four times washed fresh membranes (data not shown). These results were obtained under routine conditions of 18°C and 10 min incubation, although 2, 5, 15 and 20 min incubation yielded the same results. However, after incubating for more than 30 min the saturable (or specific) portion of bound [^{3}H]-baclofen progressively decreased with time whilst the non-specific portion increased. At

60 min the non-specific component was 16% higher than after 10 min (14348 ± 279 and 12895 ± 329 d min^{-1} mg^{-1} protein respectively, mean ± s.e.; $n = 3$ in triplicate) whereas the specific portion was 31% lower (2967 ± 287 and 4112 ± 407 d min^{-1} mg^{-1} respectively). If the incubation temperature was reduced to 4°C the specific component of bound [^{3}H]-baclofen was the same as obtained at 18°C. However, the time taken to achieve this was greater. Maximal binding occurred only after 30 min incubation. Increasing the temperature to 35°C reduced the amount specifically bound (after 10 min from 4112 ± 407 d min^{-1} mg^{-1} to 1848 ± 217 d min^{-1} mg^{-1}) at 18°C.

Ionic dependence of [^{3}H]-baclofen binding to synaptic membranes

To ascertain which ionic species present in KHS was responsible for expression of the saturable component of bound [^{3}H]-baclofen, Tris-HCl (50 mM, pH 7.4) to which the KHS salts were individually added was used as the incubation solution. The results are shown in Figure 1. A small but significant amount of saturably bound [^{3}H]-baclofen was detected in Tris-HCl buffer alone and this equalled 735 ± 91 d min^{-1} mg^{-1} protein ($n = 4$) approximately 5% of total bound compared with 3981 ± 187 d min^{-1} mg^{-1} (~21% of total) obtained in KHS ($n = 6$). When either $CaCl_2$ (2.5 mM) or $MgSO_4$ (1.2 mM) were present the saturable portion was markedly increased (3133 ± 75 and 3566 ± 47 d min^{-1} mg^{-1} protein respectively; mean ± s.e.: $n = 4$ in each case). The addition of KCl (5.9 mM) had no significant effect but NaCl (143 mM) produced a small but significant rise (128%) in the amount of [^{3}H]-baclofen specifically bound when compared with Tris-HCl buffer alone. Non-specific binding remained constant throughout.

Calcium chloride and magnesium sulphate

The amount of [^{3}H]-baclofen specifically bound to frozen and thawed synaptic membranes was dependent on the concentration of Ca^{2+} present in the Tris-HCl buffer solution. Increasing the concentration from 0 to 2.5 mM produced a progressive increase in specific binding as shown in Figure 2b. Above 2.5 mM the level declined.

A similar concentration/effect curve could be obtained with $MgSO_4$ instead of $CaCl_2$ as illustrated in Figure 2a. But in contrast to the results obtained with Ca^{2+}, the concentration of Mg^{2+} present in KHS (1.2 mM) was not optimal for maximum binding to occur. The optimal concentration appeared to be approximately 5.0 mM Mg^{2+}. The maximal amounts of binding obtained in the presence of 2.5 mM $CaCl_2$

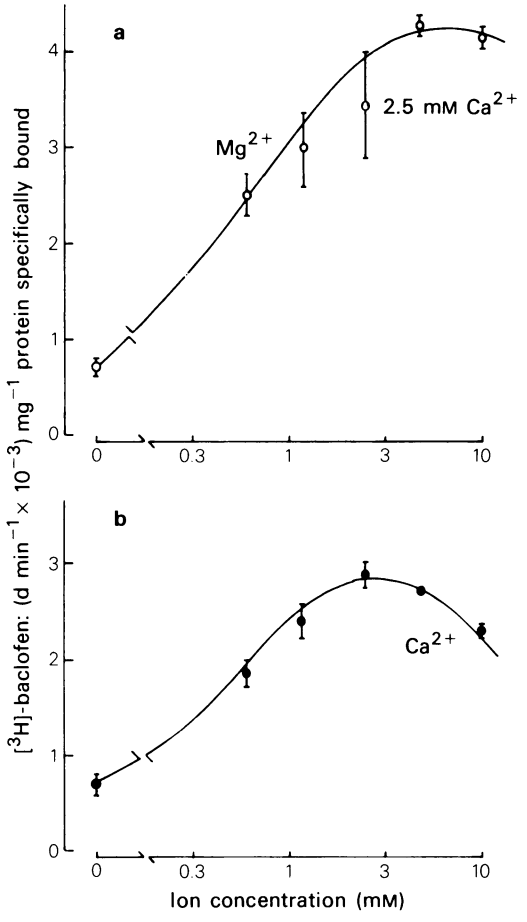


Figure 2 A comparison of the effects of increasing concentrations of the divalent cations Ca^{2+} and Mg^{2+} on the specific binding of [^{3}H]-baclofen to frozen and thawed synaptic membranes. Frozen membranes were thawed and washed twice in 50 mM Tris-HCl buffer solution alone before being resuspended and washed twice more in Tris-HCl solution containing the required concentration of cation. The pellets were finally resuspended in fresh medium with or without added cation for assay. The concentration of [^{3}H]-baclofen was 20 nM and the non-specific portion of bound ligand was determined with 100 μM (±)-baclofen. This equalled approximately 13000 d min^{-1} mg^{-1} protein at all concentrations of Ca^{2+} or Mg^{2+}. Each point represents the mean ± s.e.mean of at least three separate experiments (in triplicate) except for the value for 5 mM $CaCl_2$ which is the mean of two experiments. Increasing concentrations of (a) $MgSO_4$ (○) and (b) $CaCl_2$ (●) increased the amount of [^{3}H]-baclofen specifically bound to the membranes with maximum specific binding occurring at 2.5 mM $CaCl_2$ and 5.0 mM $MgSO_4$. In (a) the effect of a control concentration of 2.5 mM $CaCl_2$ was also determined in the same experiments. The apparent difference between the amount of saturable [^{3}H]-baclofen binding measured at 2.5 mM $CaCl_2$ and 5.0 mM $MgSO_4$ was not statistically significant.

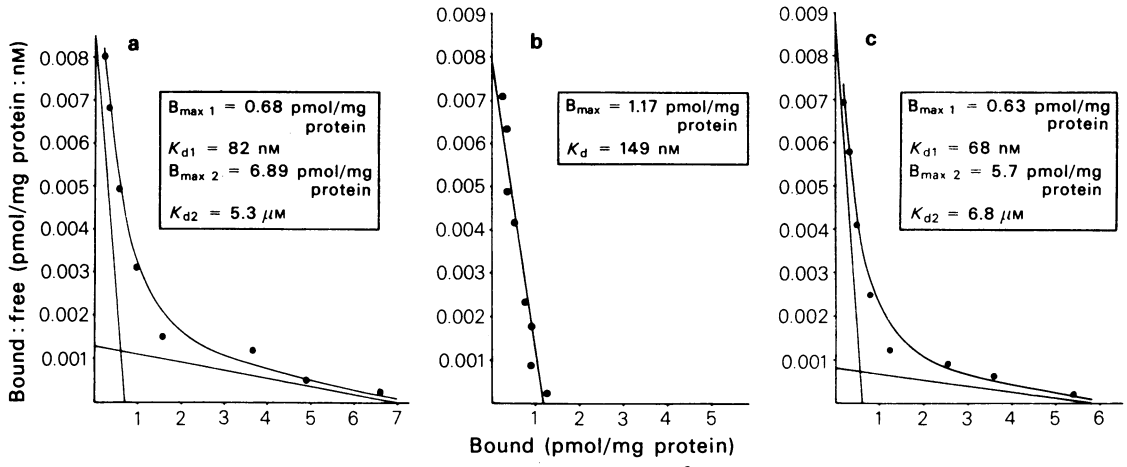


Figure 3 Comparative Scatchard analysis of the saturable binding of [^{3}H]-baclofen to frozen and thawed synaptic membranes incubated in Krebs-Henseleit solution (a) or 50 mM Tris-HCl pH 7.4 containing 2.5 mM $CaCl_2$ without (b) or with (c) 143 mM NaCl. Frozen membranes were thawed and washed four times in the appropriate solution before assay. Saturation experiments were performed using a single concentration of 20 nM [^{3}H]-baclofen together with varying concentrations of unlabelled (±)-baclofen (0.01–30 μM). Non-specific binding was determined with 100 μM (±)-baclofen. Each point represents the mean of 3–4 separate experiments performed in triplicate. In (a) and (c) the curvilinear Scatchard plots have been resolved into two components by computer analysis (see text) and the parameters used to determine the lines shown on the graph. In (b) the line has been determined by linear regression analysis.

or 5.0 mM $MgSO_4$ within the same experiments were not significantly different. The simultaneous addition of 2.5 mM $CaCl_2$ and 5.0 mM $MgSO_4$ to Tris-HCl buffer solution had no greater effect than either alone.

Saturation analysis of [^{3}H]-baclofen binding

Scatchard analysis of specifically bound [^{3}H]-baclofen in KHS yielded a curvilinear plot (Figure 3a). By contrast the same analysis of data obtained in Tris-HCl plus Ca^{2+} (2.5 mM) produced a linear plot (Figure 3b). On repeating the experiments but adding NaCl (143 mM) to the Tris-HCl plus Ca^{2+} solution produced a curvilinear plot similar to that obtained in KHS (Figure 3c). The addition of Mg^{2+} (1.2 mM) or KCl (5.9 mM) to the Tris-HCl plus Ca^{2+} solution did not alter the results from those obtained in Tris-HCl plus Ca^{2+} alone. Further analysis of the data obtained in KHS (and Tris-HCl plus Ca^{2+} plus Na^+) was performed using a non-linear least squares optimization technique (Powell, 1968) fitting them to each of 3 separate models:

(a) a simple hyperbola

$$b = \frac{B_{max} \times F}{K_d + F} \quad (1)$$

Table 1 Scatchard analysis of [^{3}H]-baclofen binding to frozen and thawed rat brain synaptic membranes: different incubation solutions

Solution	K_{d_1} (nM)	B_{max_1} (pmol/mg)	K_{d_2} (nM)	B_{max_2} (pmol/mg)
KHS – Model 1	320	3.61	—	—
Model 2*	60	1.26	—	—
Model 3	82	0.68	5300	6.9
Tris-HCl + $CaCl_2$ (2.5 mM)	149	1.17	—	—
Tris-HCl + $MgSO_4$ (1.2 mM)	126	0.98	—	—
Tris-HCl + $CaCl_2$ + $MgSO_4$	100	0.94	—	—
Tris-HCl + $CaCl_2$ + NaCl (143 mM)	68	0.63	6800	5.7
Tris-HCl + $CaCl_2$ + KCl (5.9 mM)	131	1.08	—	—

Scatchard analysis could not be performed on data obtained in Tris-HCl containing NaCl alone due to the small amount of saturable binding occurring at low ^{3}H-ligand concentrations.
* non-saturable component (pmol/mg) = 0.29

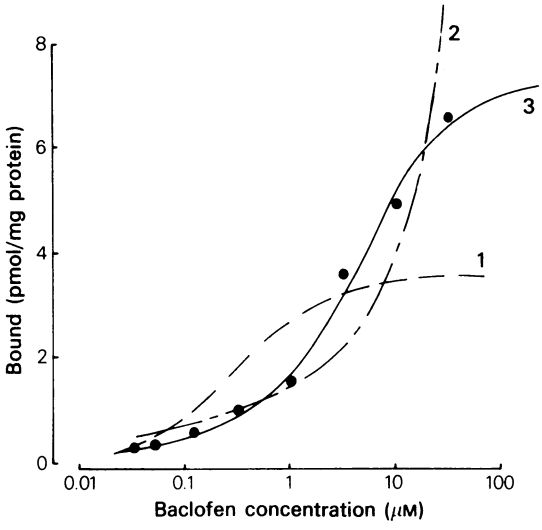


Figure 4 Comparison of binding isotherms derived for three separate theoretical models describing the curvilinear Scatchard plot observed for [^{3}H]-baclofen binding in Krebs-Henseleit solution. Frozen membranes were thawed and washed four times in Krebs-Henseleit solution. Saturable binding of [^{3}H]-baclofen was determined using a fixed concentration of the ligand (20 nM) with the addition of varying amounts of unlabelled (±)-baclofen (0.01–3 μM). Non-specific binding was determined with 100 μM (±)-baclofen. Each point is the mean of four separate experiments performed in triplicate. Each curve represents the line of optimum fit of the data to the individual models.

(1) $$b = \frac{B_{max} \times F}{K_d + F}$$

where $B_{max} = 3.61$ pmol/mg protein; $K_d = 0.32$ μM.

(2) $$b = \frac{B_{max} \times F}{K_d + F} + D \times F$$

where $B_{max} = 1.26$ pmol/mg protein; $K_d = 0.06$ μM; D (non-saturable component = 0.29 pmol/mg protein.

(3) $$b = \frac{B_{max_1} \times F}{K_{d_1} + F} + \frac{B_{max_2} \times F}{K_{d_2} + F}$$

where $B_{max_1} = 0.68$ pmol/mg protein; $K_{d_1} = 0.08$ μM; $B_{max_2} = 6.9$ pmol/mg protein; $K_{d_2} = 5.3$ μM; Ordinate b = baclofen bound (pmol/mg protein); Abscissa F = baclofen concentration (μM).

(b) a simple hyperbola plus a non-saturable component

$$b = \frac{B_{max} \times F}{K_d + F} + D \quad (1)$$

(c) two non-interacting hyperbolae

$$b = \frac{B_{max_1} \times F}{K_{d_1} + F} + \frac{B_{max_2} \times F}{K_{d_2} + F} \quad (3)$$

where b = amount bound (pmol/mg protein); B_{max} = maximum amount bound; K_d = affinity constant; F = free concentration of ligand.

The parameters which best describe the data fitted to each of these models are given in Table 1. However, it is clear from Figure 4, in which each of the isotherms described by the parameters are shown, that Model 3 provides the best approximation to the observed data. A summary of the binding parameters obtained in different incubation solutions is also given in Table 1.

Pharmacological specificity of [^{3}H]-baclofen binding site

Numerous unlabelled GABA analogues were examined for their ability to inhibit the specific binding of [^{3}H]-baclofen to synaptic membranes incubated in KHS. This incubation solution was chosen in preference to Tris-HCl plus $CaCl_2$ to enable a more direct comparison to be made with data obtained in previous experiments with rat isolated atria and mouse vas deferens (Bowery *et al.*, 1981). Results obtained with some of the analogues are shown in Figure 5. Other data have already been presented elsewhere (Hill & Bowery, 1981). A summary of the activities of the analogues is given in Table 2. Notable features of the results were: (i) Muscimol and 3-APS were only weakly active (IC_{50} values 12.3 ± 2.02 and 10.9 ± 1.05 μM respectively); (ii) (−)-Baclofen was much more active than (+)-baclofen indicating stereospecificity. The activity of the racemic mixture (±)-baclofen was between the two isomers; (iii) Isoguvacine and P4S which, like 3-APS, are potent $GABA_A$ receptor agonists were both devoid of activity at [^{3}H]-baclofen binding sites; (iv) (+)-Bicuculline methobromide (BMB), the $GABA_A$ receptor antagonist, was also inactive.

Divalent-cation dependent binding of [^{3}H]-GABA

It is generally accepted that baclofen is only very weakly active in displacing [^{3}H]-GABA from membranes incubated in Tris-citrate solution (Olsen, Greenlee, Van Ness & Ticku, 1978; Galli, Zilletti, Scotton, Adembri & Giotti, 1979; Horng & Wong, 1979; Waddington & Cross, 1979). Since specifically bound [^{3}H]-baclofen could only be detected in the presence of the divalent cations Ca^{2+} or Mg^{2+} and could be displaced by GABA, it seemed reasonable

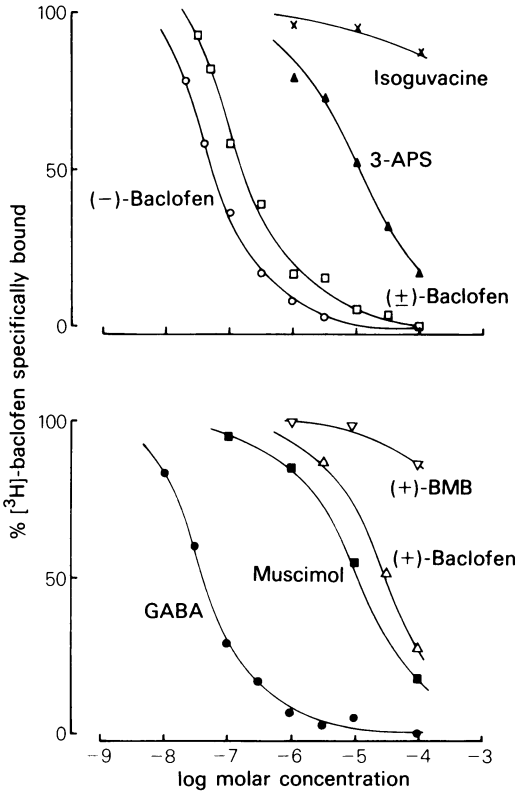


Figure 5 Inhibition by GABA analogues of the specific binding of [^{3}H]-baclofen to frozen and thawed crude synaptic membranes incubated in Krebs-Henseleit solution. Frozen and thawed membranes were washed four times in Krebs-Henseleit solution before resuspension for assay. Specific binding of 20 nM [^{3}H]-baclofen was determined with 100 μM unlabelled (±)-baclofen and in the absence of any analogue the specific portion of binding equalled 20.3 ± 0.69% of total binding (3221 ± 189 d min^{-1} mg^{-1} protein, $n = 20$). Varying concentrations of unlabelled displacers (abscissae: log molar) were added simultaneously with [^{3}H]-baclofen and the mixture incubated for 10 min at 18°C. Each point is the mean of at least three separate experiments performed in triplicate. Standard errors of the means were all less than 8% and have been omitted for clarity. The data are shown in two separate graphs also for clarity.

to test the hypothesis that a second population of baclofen-sensitive [^{3}H]-GABA binding sites might be detectable in the presence of Ca^{2+} or Mg^{2+}. Synaptic membranes prepared together in the same batch were incubated with [^{3}H]-GABA in the absence or presence of $CaCl_2$ (2.5 mM) in Tris-HCl solution. Ethylenediaminetetraacetic acid (EDTA) (5 mM) was added to the Ca^{2+}-free solution. The ability of 100 μM GABA, isoguvacine, (±)-baclofen or the combination of isoguvacine and baclofen (100 μM each) to reduce the binding of [^{3}H]-GABA was examined. The results are shown as a histogram in Figure 6. In the absence of Ca^+, GABA and isoguvacine displaced bound [^{3}H]-GABA to the same extent (> 60% displacement). By contrast (±)-baclofen, as expected, only reduced the amount bound by < 20% and the combination of baclofen and isoguvacine produced no more displacement than isoguvacine alone. EDTA did not alter the portion of [^{3}H]-GABA displaceable by isoguvacine. These results are similar to those obtained by others in Tris-citrate buffer solution (50 mM, pH 7.1). When $CaCl_2$ was substituted for EDTA the total [^{3}H]-

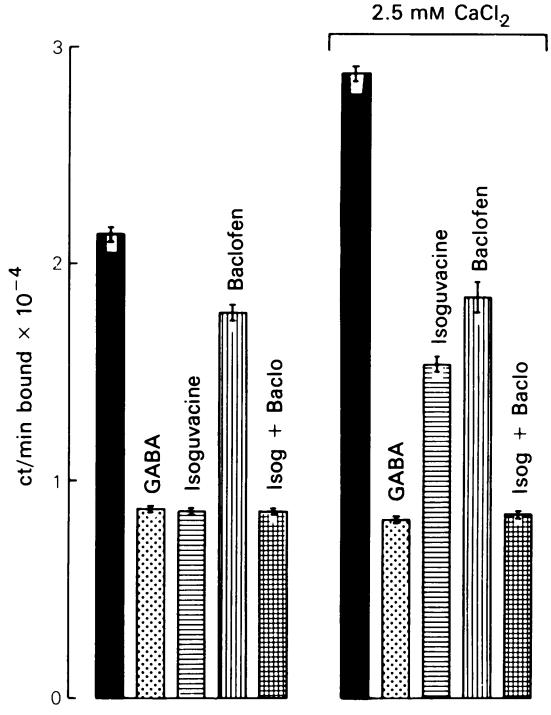


Figure 6 Inhibition of total [^{3}H]-GABA binding by GABA, isoguvacine, (±)-baclofen and isoguvacine plus baclofen in the absence and presence of 2.5 mM $CaCl_2$. Frozen membranes were thawed, washed four times and resuspended for assay in 50 mM Tris-HCl solution (pH 7.4) containing either 5.0 mM EDTA (first 5 histogram bars) or 2.5 mM $CaCl_2$ (last 5 histogram bars). The amount of [^{3}H]-GABA (10 nM) bound after 10 min at 18°C was determined in each solution alone or in the presence of 100 μM GABA, isoguvacine, (±)-baclofen or a combination of 100 μM isoguvacine plus 100 μM (±)-baclofen (Isog + Baclo). Each histogram bar represents the mean of triplicate determinations of total [^{3}H]-GABA bound (ordinate: ct/min × 10^{-4}); vertical lines show s.e.mean. Aliquots of the same membrane preparation were used throughout.

Table 2 Inhibition of specific [^{3}H]-GABA (in Tris-HCl plus Ca^{2+} plus isoguvacine 40 μM) and [^{3}H]-baclofen (in KHS) binding to $GABA_B$ sites and [^{3}H]-GABA (in Tris-citrate) to $GABA_A$ sites by related compounds in rat brain crude synaptic membranes (IC_{50} values μM ± s.e.mean)

	$GABA_B$		$GABA_A$
	[^{3}H]-GABA (10 nM)	*[^{3}H]-baclofen* (20 nM)	*[^{3}H]-GABA* (5 nM)
GABA	0.08 ± 0.01	0.041 ± 0.008	0.12 ± 0.015
(−)-baclofen	0.13 ± 0.02	0.042 ± 0.009	IA
(±)-baclofen	0.13 ± 0.05	0.184 ± 0.028	IA
SL75102	0.50 ± 0.05†	0.40 ± 0.02†	1.4*
β-*o*-chlorophenyl GABA	0.92 ± 0.08	0.74 ± 0.34	IA
β-hydroxy GABA	1.10 ± 0.10	1.60 ± 0.15	1.38 ± 0.15
β-*p*-fluorophenyl GABA	1.70 ± 0.17	1.60 ± 0.001	IA
β-chloro GABA	4.6 ± 1.1	11.4 ± 1.69	0.69 ± 0.18
Muscimol	5.4 ± 0.71	12.3 ± 2.02	0.015 ± 0.003
β-*m*-chlorophenyl GABA	19.1 ± 0.93	17.7 ± 0.3	IA
β-phenyl GABA	9.6 ± 0.37	IA	IA
3-Aminopropanesulphonic acid	10.0 ± 0.10	10.9 ± 1.05	0.11 ± 0.015
δ-Aminovaleric acid	30	50	5.0 ▽
(+)-Baclofen	74.0 ± 5.7	33.3 ± 3.0	IA
SL76002	IA†	IA†	35*
β-napthyl GABA	IA	IA	IA
γ-Hydroxy butyric acid	IA	IA	IA
THIP	IA	IA	0.45 ▽
p-isopropyl phenyl GABA	IA	IA	IA
Isoguvacine	IA	IA	0.19 ± 0.09
Piperidine-4-sulphonic acid	IA	IA	
(+)-bicuculline methobromide	IA	IA	27.6 ± 3.67
(−)-bicuculline methobromide	IA	IA	IA
Picrotoxin	IA	IA	IA
(±)-*cis*-3-Aminocyclohexane carboxylic acid	IA	IA	IA
(±)-Nipecotic acid	IA	IA	IA

IC_{50} values determined by probit analysis of data from 3 or more experiments for each analogue (concentration range 10 nM–100 μM; triplicate determinations at each concentration within any experiment). IA = $IC_{50} > 100$ μM
† from Bowery, Hill & Hudson (1982a)
* from Bartholini, Scatton, Zivkovic & Lloyd (1979)
▽ from Olsen, Ticku, Greenlee & Van Ness (1979)
SL75102 = [α(4-chlorophenyl)5-fluoro 2-hydroxy benzilidene-amino]-4-butanoate sodium
SL76002 = [α(4-chlorophenyl)5-fluoro 2-hydroxy benzilidene-amino]-4-butyramide
THIP = 4,5,6,7-tetrahydroisoxazolo [5,4-c]pyridin-3-ol

GABA bound to the tissue was enhanced by approximately 35% whereas non-specific binding as estimated by 100 μM GABA did not change. The residual binding in the presence of 100 μM GABA was the same irrespective of the incubation medium. Isoguvacine, however, only displaced the same amount of [^{3}H]-GABA in the presence of Ca^{2+} as in its absence. The calcium-dependent portion of bound [^{3}H]-GABA was completely suppressed by (±)-baclofen (100 μM). The combination of isoguvacine and baclofen now displaced the same amount as GABA alone.

These results indicate that in the presence of 2.5 mM $CaCl_2$, [^{3}H]-GABA labels two separate populations of binding site. One is sensitive to isoguvacine but not baclofen. The other is dependent upon the presence of divalent cation and is sensitive to baclofen but not isoguvacine.

Saturation analysis of divalent cation-dependent [^{3}H]-GABA binding

Saturation analysis of the specific binding of [^{3}H]-GABA to baclofen-sensitive sites was performed on synaptic membrane preparations treated in three different ways: (a) freshly prepared and washed four times but not frozen; (b) frozen, thawed and four times washed; or (c) frozen, thawed, washed four times and incubated in Triton-X-100 (0.05% v/v) for 30 min at 35°C.

In all the incubations with [^{3}H]-GABA (10 nM), isoguvacine (40 μM) was present to suppress any

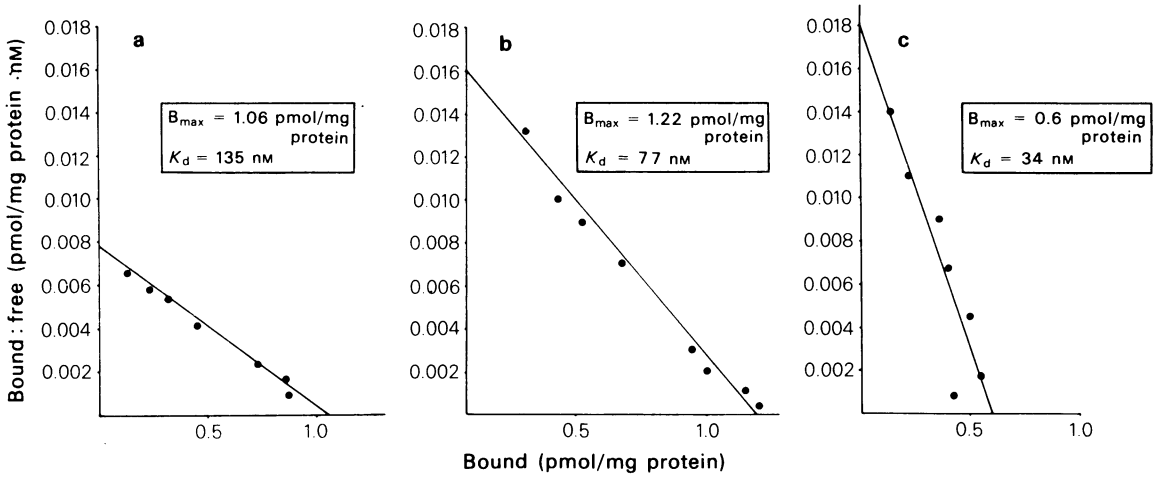


Figure 7 Scatchard analysis of saturable [^{3}H]-GABA binding to $GABA_B$ sites on fresh, frozen and thawed or frozen and Triton-treated synaptic membranes. Crude synaptic membranes were prepared as described in Methods and (a) washed four times in 50 mM Tris-HCl buffer pH 7.4 containing 2.5 mM $CaCl_2$; (b) frozen for at least 16 h (−20°C) before thawing and washing four times in 50 mM Tris-HCl plus Ca^{2+}; or (c) frozen for at least 16 h before thawing and washing once in Tris-HCl plus Ca^{2+} followed by incubation in 0.05% v/v Triton-X-100 (in Tris-HCl plus Ca^{2+}) for 30 min at 35°C. After incubation in Triton the membranes were centrifuged at 48000 *g* for 20 min and washed twice by resuspension in fresh buffer and centrifugation at 48000 *g* for 20 min. For the binding assay the membrane pellets in each case (a), (b) and (c) were resuspended in Tris-HCl plus Ca^{2+} solution containing 40 μM isoguvacine. The saturable binding of a fixed concentration (10 nM) of [^{3}H]-GABA was determined over a range of concentrations (0.01–3 μM) of unlabelled GABA added simultaneously. Non-specific binding was estimated by 100 μM (±)-baclofen. Each point is the mean of 3–4 experiments performed in triplicate. The binding data yielded linear Scatchard plots in all 3 cases consistent with binding to a single population of sites.

binding to $GABA_A$ sites and $CaCl_2$ (2.5 mM) was present to promote binding to $GABA_B$ sites.

(a) *Fresh membranes:* in these membranes saturable binding represented 36 ± 1.5% (mean ± s.e.: $n = 3$) of the total bound. This was equivalent to 9948 ± 946 d min^{-1} mg^{-1} protein. Scatchard analysis yielded a linear plot consistent with binding to a single homogeneous population of binding sites with $K_d = 135$ nM, $B_{max} = 1.06$ pmol/mg protein (Figure 7a).

(b) *Frozen membranes:* freezing and thawing the membrane preparation produced a significant increase of 109% in the amount of [^{3}H]-GABA specifically bound to $GABA_B$ sites (20851 ± 1180 d min^{-1} mg^{-1} protein, $n = 6$) when compared with fresh tissue. The specific component equalled 44.8 ± 2.9% of total bound [^{3}H]-GABA Scatchard analysis of these data (Figure 7b) indicated that the change in specific binding resulted from an increase in affinity ($K_d = 77$ nM) of the binding sites for GABA rather than any change in the maximum number of sites (1.22 pmol/mg protein). Only one binding site was evident. The change in affinity produced by freezing may be due to the removal of an interfering substance(s).

(c) *Triton-treated membranes:* the amount of [^{3}H]-GABA specifically bound to membranes after incubation in Triton-X-100 was, if anything, reduced from that obtained after freezing and thawing alone (16577 ± 1529 d min^{-1} mg^{-1} protein, $n = 6$) although the specific component still represented 42.0 ± 2.3% of the total [^{3}H]-GABA bound. However, Scatchard analysis revealed a clear difference between the treated and untreated membranes (Figure 7c). Triton-treatment significantly reduced the binding capacity by approximately 50% to 0.6 pmol/mg protein whilst raising the affinity of these sites by a factor of 2 ($K_d = 34$ nM).

Influence of [$CaCl_2$] on the binding of [^{3}H]-GABA to $GABA_B$ sites

The amount of [^{3}H]-GABA bound to $GABA_B$ sites was maximal in the presence of 2.5 mM $CaCl_2$ (data not shown). As with [^{3}H]-baclofen at concentrations above or below this the amount bound was reduced. The ED_{50} was approximately 0.2 mM $CaCl_2$. Scatchard analysis of [^{3}H]-GABA binding to $GABA_B$ sites in the presence of 0.6 mM $CaCl_2$ (Figure 8) indicated that the lower amount of specifically bound ligand resulted from a decrease in the maximum number of binding sites and not a change in affinity. The B_{max}

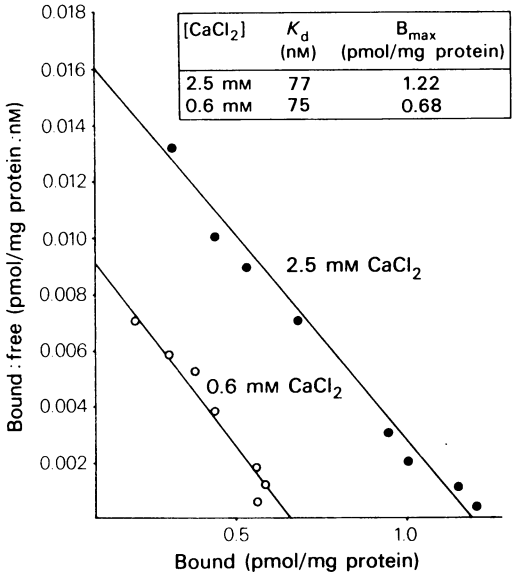


Figure 8 Scatchard analysis of [^{3}H]-GABA bound to $GABA_B$ sites on rat synaptic membranes in the presence of 2.5 mM and 0.6 mM $CaCl_2$. Membranes were thawed then washed four times and incubated in 50 mM Tris-HCl (pH 7.4) containing either 2.5 mM (●) or 0.6 mM (○) $CaCl_2$. Experiments were performed with a fixed amount of [^{3}H]-GABA (10 nM) and varying concentrations of unlabelled ligand (0.01–3 μM). Non-specific binding was determined with 100 μM (±)-baclofen. Each closed circle represents the mean of 6 experiments and each open circle the mean of 3 experiments, performed in triplicate. The linear regression line for each set of transformed data is shown together with the binding constants derived from it.

was reduced from 1.22 to 0.68 pmol/mg protein whereas the K_d values were 77 nM and 75 nM respectively.

Pharmacological specificity of divalent cation dependent [^{3}H]-GABA binding

The same unlabelled GABA analogues studied as displacers of [^{3}H]-baclofen were also examined for their ability to displace [^{3}H]-GABA from $GABA_B$ sites (in Tris-HCl solution containing $CaCl_2$ 2.5 mM and isoguvacine 40 μM). GABA, (−)-baclofen and (±)-baclofen all suppressed the amount of bound [^{3}H]-GABA. The degree of displacement was dose-related and paralleled that produced by increasing concentrations of GABA (Hill & Bowery, 1981). Muscimol, 3-APS and (+)-baclofen were again only weakly active and (+)-BMB (100 μM) produced $< 10\%$ displacement. A summary of the comparative potencies (IC_{50}s) of the analogues is given in Table 2 alongside their ability to displace [^{3}H]-baclofen. The IC_{50} values for each analogue were strikingly similar in both assays with one exception, β-phenyl GABA, which was 10–20 times more potent as an inhibitor of [^{3}H]-GABA binding than of [^{3}H]-baclofen. The weaker effect against [^{3}H]-baclofen was also apparent in Tris-HCl buffer plus Ca^{2+}; thus the discrepancy was not due to the different incubation solutions.

For comparison, the analogue concentrations required to displace [^{3}H]-GABA by 50% from $GABA_A$ sites are also shown in Table 2. Clearly the values differ from those obtained in the $GABA_B$ site assays.

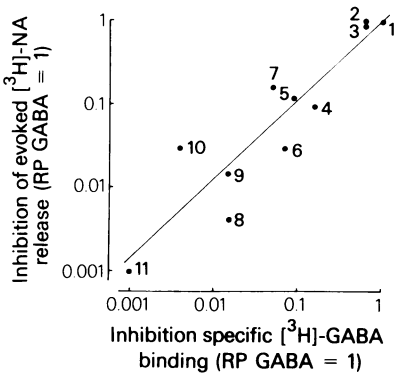
Correlation between data from binding and in vitro *assays*

A comparison was made between the relative potencies of the analogues (GABA = 1) in displacing [^{3}H]-GABA or [^{3}H]-baclofen from $GABA_B$ sites and their activity as inhibitors of the evoked release of [^{3}H]-noradrenaline from rat isolated atria or inhibitors of the twitch response of guinea-pig or mouse isolated vas deferens. Correlograms illustrating this are shown in Figures 9 and 10. In Figure 9 [^{3}H]-GABA displacement is compared with inhibition of atrial [^{3}H]-noradrenaline release and in Figure 10 [^{3}H]-baclofen displacement is compared with the reduction in vas deferens twitch response. A positive correlation is evident in both cases with coefficients of 0.91 and 0.73 respectively.

Discussion

We have shown that [^{3}H]-baclofen and [^{3}H]-GABA label sites ($GABA_B$ sites) in the rat brain which appear to be distinct from previously described binding sites for [^{3}H]-GABA and its analogues (Zukin *et al.*, 1974; Enna & Snyder, 1975; Beaumont Chilton, Yamamura & Enna, 1978; Snodgrass, 1978; Wang, Salvaterra & Roberts, 1979; Collins, McDonald & Newton, 1980; Morin & Wasterlain, 1980; Krogsgaard-Larsen, Snowman, Lummis & Olsen, 1981; Falch & Krogsgaard-Larsen, 1982).

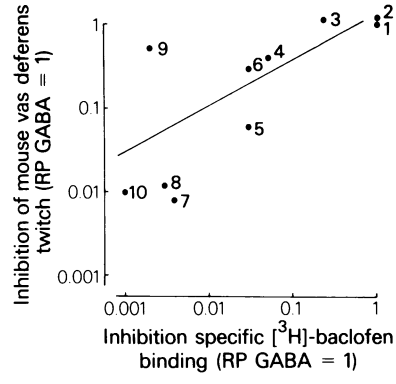
This distinction is evident from the absolute dependence on divalent cations exhibited by $GABA_B$ sites and from the different pharmacological profiles of the binding sites. The binding of ligands to 'classical' GABA Na^+-independent receptor sites ($GABA_A$ sites) appears to be independent of the presence of Ca^{2+} or Mg^{2+} (Enna & Snyder, 1977, and present observations). By contrast, the absence of these ions prevents detection of any binding to $GABA_B$ sites. Pharmacological separation of the sites is apparent from the inability of certain potent $GABA_A$ site agonists to displace radiolabelled



1 GABA
2 (−)-Baclofen
3 (±)-Baclofen
4 SL75102
5 (±)-β-*o*-chloro phenyl GABA
6 (±)-β-hydroxy GABA
7 (±)-β-*p*-fluoro phenyl GABA
8 (±)-β-chloro GABA
9 Muscimol
10 (±)-β-*m*-chloro phenyl GABA
11 (+)-Baclofen

Figure 9 Correlation of the potencies of GABA analogues in inhibiting the specific binding of [^{3}H]-GABA to $GABA_B$ sites in rat synaptic membranes (abscissa scale) and the evoked release of [^{3}H]-noradrenaline ([^{3}H]-NA) from rat isolated atria (ordinate scale). The data for inhibition of specific [^{3}H]-GABA (10 nM) binding to $GABA_B$ sites were obtained using previously frozen crude synaptic membranes washed four times and incubated in 50 mM Tris-HCl plus $CaCl_2$ (2.5 mM) solution containing 40 μM isoguvacine with or without displacer (0.01–100 μM). Concentrations of the displacer producing 50% inhibition of specifically bound [^{3}H]-GABA were determined from probit analysis of data from at least three experiments for each compound (Table 2). These values were used to calculate the potency of each compound relative to GABA (IC_{50} GABA = 0.08 μM = 1).

Relative potencies (RP) for inhibition of the evoked release of [^{3}H]-noradrenaline from rat atria were taken from Bowery *et al.* (1981) (ED_{50} GABA = 4.2 μM = 1). Correlation coefficient = 0.91 ($P < 0.001$).



1 GABA
2 (−)-Baclofen
3 (±)-Baclofen
4 (±)-β-*o*-chloro phenyl GABA
5 (±)-β-hydroxy GABA
6 (±)-β-*p*-fluoro phenyl GABA
7 (±)-β-chloro GABA
8 Muscimol
9 (±)-β-*m*-chloro phenyl GABA
10 (+)-Baclofen

Figure 10 Correlation of the relative potencies of GABA analogues in inhibiting the specific binding of [^{3}H]-baclofen to rat crude synaptic membranes (abscissa scale) and the twitch response of the mouse isolated vas deferens (ordinate scale). The data for inhibition of specific [^{3}H]-baclofen binding were obtained as indicated in Figure 9 from the IC_{50} values shown in Table 2 (IC_{50} GABA = 0.04 μM = 1). Relative potencies for depression of the twitch response of the mouse vas deferens were taken from Bowery *et al.* (1981). The potencies relative to GABA of the 10 analogues tested in these two assay systems showed a poor but significant correlation $r = 0.73$ ($P < 0.05$).

ligands from $GABA_B$ sites. For example, whilst isoguvacine, P4S and THIP are potent displacers at $GABA_A$ sites (Krogsgaard-Larsen, Johnston, Lodge & Curtis, 1977; Krogsgaard-Larsen & Arnt, 1980; Krogsgaard-Larsen, Falch, Schousboe, Curtis & Lodge, 1980), these compounds are completely devoid of activity at $GABA_B$ sites. By comparison (−)-baclofen is a potent agonist at $GABA_B$ sites but is virtually inactive in displacing [^{3}H]-GABA from $GABA_A$ sites (Olsen *et al.*, 1978; Galli *et al.*, 1979; Horng & Wong, 1979). The separation is further supported by the inactivity of the GABA antagonist (+)-bicuculline and its methohalide salts at $GABA_B$ sites; cf. $GABA_A$ sites (Zukin *et al.*, 1974; Enna & Snyder, 1975; Collins & Cryer, 1978).

The pharmacological characteristics of $GABA_B$ binding sites compare favourably with those previously described in intact peripheral preparations (Bowery *et al.*, 1981) and with data from release studies in brain slices (Bowery *et al.*, 1980b; Bowery, Doble, Hill, Hudson & Turnbull, 1980a). Thus the bicuculline-insensitive, baclofen-sensitive $GABA_B$ receptor exists within as well as outside the mammalian CNS. The apparent differences between this recognition site and 'classical' $GABA_A$ receptors are summarized in Table 3. Reports from other laboratories as well as from our own have already shown that $GABA_B$ receptors are widely distributed in the mammalian periphery. So far receptors with $GABA_B$ characteristics have been described in intestinal muscle (Kaplita, Waters & Triggle, 1982), atria (Bowery *et al.*, 1981) vas deferens (Bowery *et al.*, 1981; Stone, 1981), vascular muscle (Starke & Weitzell, 1981) and anoccocygeus muscle (Hughes, Mor-

Table 3 Differences between $GABA_A$ and $GABA_B$ site characteristics

$GABA_A$ sites	*$GABA_B$ sites*
Isoguvacine agonist	Isoguvacine inactive
P4S agonist	P4S inactive
(−)-Baclofen inactive	(−)-Baclofen agonist
Bicuculline antagonist	Bicuculline inactive
GTP no effect	GTP decreases binding affinity (Bowery, Hill & Hudson, 1982b)
Muscimol potent agonist	Muscimol very weak agonist
Linked to Cl^- channels	Not linked to Cl^- channels
Triton-X-100 treatment increases binding capacity	Triton-X-100 treatment decreases binding capacity
Cerebellar location mainly granule cell layer	Cerebellar location only molecular layer
Ca^{2+}/Mg^{2+} independent	Ca^{2+}/Mg^{2+} dependent

gan & Stone, 1982; Muyhaddin, Roberts & Woodruff, 1982a). They have also been demonstrated in the basilar artery (Anwar & Mason, 1982). In all cases the indications are that the receptor is located on nerve terminal inputs to the tissues. Activation of the receptor diminishes evoked transmitter release reducing the postsynaptic response. This site of action is emphasized by studies on labelled neurotransmitter release in the atrium (Bowery *et al.*, 1981), vas deferens (Bowery & Hudson, 1979) and anococcygeus muscle (Muhyaddin, Roberts & Woodruff, 1982b).

It is possible, therefore, that the binding sites on brain membranes are at least in part located presynaptically. However, we have no information at present to support or refute this. $GABA_B$ sites can be detected in many areas of the mammalian brain (Wilkin, Hudson, Hill & Bowery, 1981; Horton & Sykes, 1982; Lloyd, Arbilla, Beaumont, Briley, deMontis, Scatton, Langer & Bartholini, 1982) and autoradiographical analysis indicates that their regional location can differ from that of $GABA_A$ sites (Wilkin *et al.*, 1981; Price, Wilkin & Bowery, unpublished observations). For example, the molecular layer of the cerebellum is rich in $GABA_B$ sites but as pointed out by Wilkin *et al.* (1981), if $GABA_B$ sites are functional they are unlikely to be present on presynaptic sites since there is no evidence of any axo-axonic contacts in this brain region. It is possible that GABA released at axo-somatic contacts may 'wash-over' onto presynaptic sites in close proximity but a postsynaptic location seems more plausible. Thus, like bicuculline-sensitive GABA receptors, (Curtis & Johnston, 1974; Curtis, 1978), $GABA_B$ sites may exist at pre- and postsynaptic sites depending on the brain region.

It is generally believed that baclofen produces its central depressant effects by reducing transmitter release rather than by a direct postsynaptic action (Davidoff & Sears, 1974; Ault & Evans, 1978; Fox, Krnjević, Morris, Puil & Werman, 1978; Potashner, 1979; Curtis, Lodge, Bornstein & Peet, 1981; Davies, 1981). It is not known whether $GABA_B$ sites are implicated in the central depressant effect but the (−)-isomer is the active form not only in binding studies but also in depressing neuronal activity (Olpe, Demieville, Baltzer, Bencze & Koella, 1978). However, the action of baclofen may not be confined to $GABA_B$ receptors. Perhaps these receptors are responsible for only some of the effects of the drug.

The importance of Ca^{2+} or Mg^{2+} in the binding of [^{3}H]-GABA or [^{3}H]-baclofen to $GABA_B$ sites is particularly interesting in the light of recent findings by Dunlap (1981) and Feltz and colleagues (Desarmenien, Feltz, Loeffler, Occhipinti & Santangelo, 1982) who have shown that $GABA_B$ site activation diminishes Ca^{2+} conductance in normal or cultured dorsal root ganglion neurones. The requirement for divalent cations in the binding of the ligand and the change in cation conductance resulting from the receptor occupation are not necessarily associated. Nevertheless, it is an attractive idea that the two phenomena may be interrelated. If the binding process decreases available Ca^{2+} this might diminish transmembrane movement. The extent to which other divalent cations can substitute for Ca^{2+} or Mg^{2+} in the binding process is currently under investigation. It is perhaps significant that the optimal concentrations of Ca^{2+} and Mg^{2+} required to achieve maximal binding to $GABA_B$ sites coincide with the extracellular physiological concentrations of these ions.

The small Na^+-dependent component of [^{3}H]-baclofen binding is clearly additional to that obtained in the presence of Ca^{2+} or Mg^{2+} alone. However, the pharmacological specificity of this site appears to be the same as the Ca^{2+}-dependent component. It seems unlikely that it is associated with a transport recognition site since [^{3}H]-baclofen was not accumulated by brain slices incubated under conditions in which [^{3}H]-GABA was avidly taken up. It has previously been shown by others that baclofen does not

inhibit the accumulation of [^{3}H]-GABA by brain slices (Tardy, Rolland, Bardakdjian & Gonnard, 1978).

$GABA_B$ binding sites could be detected on freshly prepared tissue but freezing the membranes increased their affinity for the ligand. This result is analogous to the effect of freezing on bicuculline-sensitive binding sites (Enna & Snyder, 1975). It appears that freezing and thawing removes proteins and/or phospholipids present in the membrane preparation (Johnston & Kennedy, 1978; Toffano, Guidotti & Costa, 1978; Yoneda & Kuriyama, 1980). These normally impair the binding of [^{3}H]-GABA. GABA itself could account for a portion of the inhibitory factor (Napias, Bergman, Van Ness, Greenlee & Olsen, 1980; Gardner, Klein & Grove, 1981). Replacement of the supernatant obtained during the freezing and thawing procedures into the incubation mixture reduces the amount bound suggesting that an inhibitory material can be extracted (Johnston & Kennedy, 1978). Perhaps a similar extraction occurs in relation to membrane $GABA_B$ sites even if it is only GABA.

Freezing reduces the Na^+-dependent component of total bound [^{3}H]-GABA. This component is thought to represent binding to transport recognition sites (Enna, 1981). The observation that specifically bound [^{3}H]-baclofen was greater in frozen and thawed membranes than in fresh tissue suggests that the Na^+-dependent component does not represent binding to membrane transport sites and provides further support for a receptor recognition site.

Binding to $GABA_A$ sites is increased if the membranes are treated with Triton-X-100 (Enna & Snyder, 1977). Prior incubation with this non-ionic detergent increases the binding affinity and the density of binding sites. Such treatment, like freezing, is thought to remove endogenous inhibitors (Johnston & Kennedy, 1978), since again replacement of the extraction solution reduces the binding parameters. $GABA_B$ binding sites did not show the same response to Triton treatment. Whilst the binding affinity may have shown a small increase, the number of binding sites was actually reduced by 50%. This would suggest that $GABA_B$ receptors may be more labile than $GABA_A$ sites and Triton detergent solubilizes the receptors and/or cation binding sites out of the membranes. However, we have not yet measured specific [^{3}H]-GABA binding in the supernatant following Triton treatment and thus cannot rule out the possibility that the detergent effects could be due to denaturation of the receptors.

In conclusion, $GABA_B$ sites can be readily demonstrated in mammalian brain tissue by radiolabelled binding techniques using [^{3}H]-baclofen or [^{3}H]-GABA as the ligand. The characteristics of this binding site support the view that it is a receptor recognition site analogous to that described in intact peripheral systems (Bowery *et al.*, 1981) and show that it is distinct from bicuculline-sensitive $GABA_A$ receptors. Whether $GABA_B$ sites are functional receptors remains to be determined. Studies in this direction would be facilitated with the availability of a selective antagonist. Muhyaddin *et al.* (1982a) have shown that δ-aminovaleric acid is an antagonist at $GABA_B$ receptors. However, it is only very weakly active and is more potent as an agonist at $GABA_A$ receptors. At $GABA_A$ binding sites in rat brain its potency relative to GABA = 0.04 (Olsen *et al.*, 1979) and in depolarizing sympathetic ganglia 0.05 (GABA = 1) (Bowery & Brown, 1974). By comparison, its potency in displacing ligands from $GABA_B$ sites was approximately 0.001. However, as pointed out by Muhyaddin *et al.* (1982a) structural analogues of this compound may prove to be of greater potency and selectivity.

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