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BINDING OF [^{3}H]-MUSCIMOL, A POTENT γ-AMINOBUTYRIC ACID RECEPTOR AGONIST, TO MEMBRANES OF THE BOVINE RETINA

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1 The binding of [^{3}H]-muscimol, a potent γ-aminobutyric acid (GABA) receptor agonist, to crude membrane preparations of bovine retina was studied, using a filtration method to isolate membrane-bound ligand.

2 Specific binding was found to be saturable and occurred at two binding sites with affinity constants of 4.3 nM and 38.2 nM.

3 Binding was sodium-independent, enhanced by both freezing and Triton X-100 treatment but abolished with sodium laurylsulphate.

4 The binding sites demonstrated a high degree of pharmacological specificity, GABA being a potent displacer of [^{3}H]-muscimol.

5 A higher degree of [^{3}H]-muscimol binding was associated with subcellular fractions enriched with photoreceptor synaptosomes rather than with fractions enriched with conventional synaptosomes.

Introduction

There is substantial evidence that γ-aminobutyric acid (GABA) is a synaptic neuro-transmitter substance in the vertebrate retina (see Graham 1974 for review). It has been demonstrated that GABA is synthesized and stored in certain neurones in the retina and that the intracellular level can be altered by light (Lam, 1972; 1978). A very important criterion of whether a substance is a transmitter is to show the existence of physiologically relevant receptor sites. Using specific radioactive [^{3}H]-GABA, Enna & Snyder (1976) and Redburn, Kyles & Ferkany (1979) were able to demonstrate the existence of GABA binding sites in the retina. These binding sites are, however, thought to represent not only the postsynaptic receptors for GABA, but also uptake sites.

More recently, the potent GABA receptor agonist, muscimol, has become available as a radioligand and has been used by a number of workers to characterize the synaptic GABA receptor in the mammalian brain (Beaumont, Chilton, Yamamura & Enna, 1978; Snodgrass, 1978; Williams & Risley 1979). Muscimol has a higher affinity for the GABA receptor than GABA itself and apparently labels the receptor rather than the uptake sites (see Johnston, 1978; Williams & Risley, 1979).

In the present study, a rapid filtration assay has been used to characterize the binding of [^{3}H]-muscimol to membranes of the bovine retina. Subcellular fractions of the retina were also prepared and analysed, in order to determine the subcellular localization of retinal GABA receptors.

Methods

Bovine eyes were collected from the abattoir (British Beef Co., Witney, Oxfordshire) within 10 min of the animal's death. The retinas were then placed in Krebs-phosphate medium (Dawson, Elliot, Elliott & Jones, 1972) at 2°C, containing 0.1 mM ascorbate and transported to the laboratory, where they were rinsed in cold Krebs-bicarbonate medium (Dawson, *et al.* 1972) and either weighed out in 1 g portions and kept in the deep freeze until further use or fractionated into various components.

Frozen retinas were subsequently homogenized (1 g/100 ml) in cold 0.05 M Tris/HCl buffer pH 7.4, in a Teflon grinder with a glass pestle rotating at 900 rev/min with 6 up and down strokes. After centrifuging the homogenates at 25,000 *g* for 30 min, the supernatant was discarded and the pellet resuspended in the same volume of Tris/HCl as above and centrifuged a second time at 25,000 *g* for 30 min. The resultant pellet was resuspended in 1/10th of the original volume of ice-cold Tris/HCl buffer and immediately assayed. Usually, 200 μl of the membrane preparation was incubated in 0.05 M Tris/HCl buffer pH 7.4 with various concentrations (0.5 to 300 nM) of [^{3}H]-musci-

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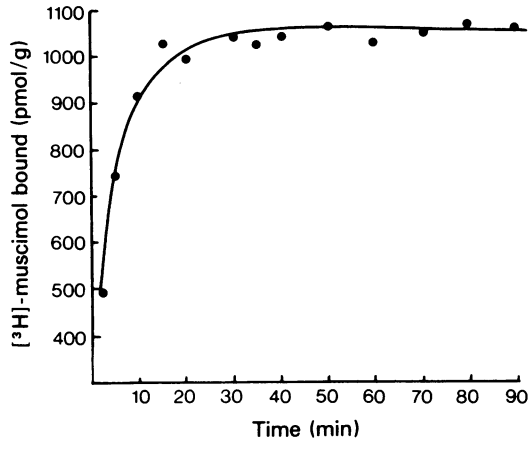

Figure 1 Time course of specific [^{3}H]-muscimol binding to membranes prepared from bovine retina. [^{3}H]-muscimol concentration was 5 nM, incubation temperature was 4°C. Specific binding is defined as the difference between total [^{3}H] muscimol binding and non-specific binding (where 10^{-4} M GABA was also present). Points are mean values of four experiments; each experiment was carried out in triplicate. Percentage error varies from 5 to 10%.

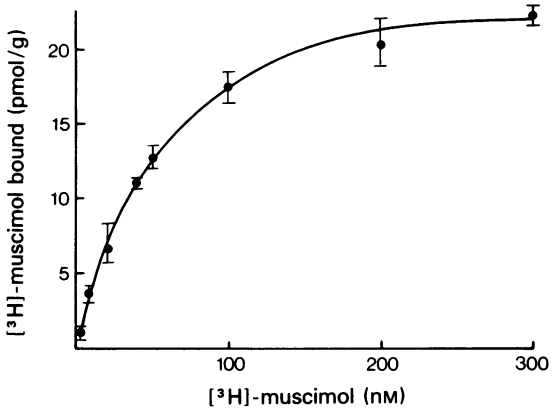

Figure 2 Specific binding of various concentrations of [^{3}H]-muscimol to retinal membrane preparations for a period of 30 min at 4°C. Each point is the mean of at least three experiments each carried out in triplicate; vertical lines show s.d.

mol (New England Nuclear, specific activity 13.7 Ci/mmol) in the absence (total binding) or presence (non-specific binding) of 10^{-4}M GABA. The final volume was 0.5 ml. After incubation at 4°C for 30 min, the reaction was terminated by the addition of 5 ml of ice-cold Tris-HCl buffer, the sample rapidly filtered onto Whatman GF/B glass fibre filters and washed on the filter with 2 × 5 ml aliquots of ice-cold 0.05 M Tris/HCl buffer. Filters were placed in 10 ml scintillation fluid (water: Triton X-100: toluene, 1:2:4 by vol, plus 0.04% PPO and 0.01% POPOP) and counted at an efficiency ranging between 20 and 27%. Specific binding was determined as the difference in the ct/min bound to filters in the absence and presence of GABA and represented approximately 85% of the total counts. Thin layer chromatography on silica precoated plates (Merck), using an ethyl acetate:isopropanol:ammonium hydroxide (9:7:5:by vol) system indicated that about 95% of membrane-bound isotope was unchanged [^{3}H]-muscimol.

When analysing the influence of Triton X-100 and sodium laurylsulphate (SDS), membranes were initially incubated with the substances in Tris/HCl for 15 min at room temperature. The membrane preparations were then recovered by centrifugation at 25,000 *g* for 30 min and resuspended in Tris/HCl containing [^{3}H]-muscimol. The binding of the [^{3}H]-muscimol was then analysed in the usual way.

Fresh retinas were also hand-homogenized in 0.32 M sucrose (pH 7.1) with a glass-Teflon tissue grinder and fractionated as described by Redburn (1977). Two populations of synaptosomes, one highly enriched in photoreceptor nerve endings (P_1) and the other containing synaptosomes derived from the inner plexiform layer (P_2), were produced, suspended in 0.05 M Tris/HCl buffer, homogenized and assayed for [^{3}H]-muscimol binding.

IC_{50}s for the various compounds examined were determined by running 3 to 6 concentrations of each compound in triplicate. Protein determinations were carried out by the method of Lowry, Rosebrough, Farr & Randall (1951).

Results

Binding of [^{3}H]-muscimol to membranes prepared from retina stored at −20°C was stable for up to 30 days. Binding was independent of sodium up to 150 mM, was increased by 50 ± 8% (±s.d., n = 5) following treatment with 0.05% Triton X-100 and destroyed by heating to 100°C or treatment with 0.05% SDS. Binding of [^{3}H]-muscimol to fresh retina preparations, as opposed to frozen material, was diminished by 30 ± 10% (±s.d., n = 3).

The association of [^{3}H]-muscimol with its binding site in bovine retinal membranes was rapid, as shown in Figure 1. Owing to the limitation of the filtration techniques, it was not possible to examine binding accurately at intervals of less than 2.5 min, but even at these times binding had reached a level approximately a half of the eventual maximum. Equilibrium was reached at about 30 min after the start of the incubation, so this time was routinely chosen for the incubation in subsequent experiments.

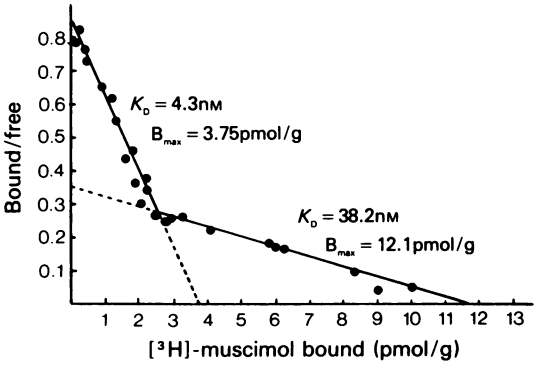

Figure 3 Scatchard plot of [^{3}H]-muscimol to bovine retinal membranes. Membranes were incubated with various concentrations of [^{3}H]-muscimol as described in Methods. The data in the curve shown above were from two separate experiments carried out in triplicate.

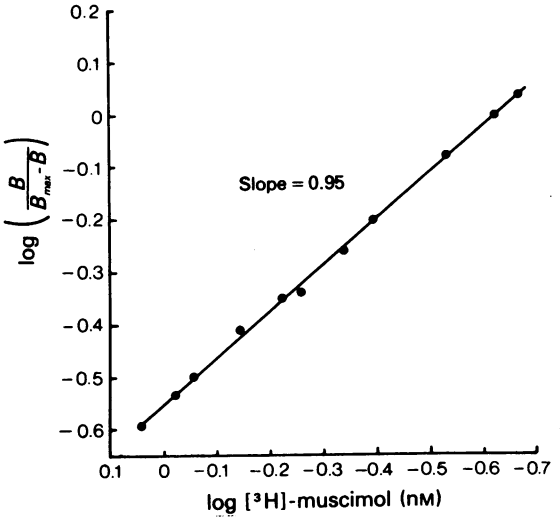

Figure 4 Hill plots using the data for the high affinity binding site (see Figure 3) of [^{3}H]-muscimol to retinal membranes. The line of best fit was estimated by the method of least squares (r = 0.95).

As seen from Figure 2, the specific binding isotherm for various concentrations of [^{3}H]-muscimol to retinal membrane preparations for a period of 30 min is a hyperbolic curve, which approaches saturation at [^{3}H]-muscimol concentrations above 200 nM.

Non-specific binding (which was linear with concentration) was subtracted from the total binding to give the specific binding isotherm (Figure 2). Scatchard analysis (see Bennett, 1978) of the binding data, using a least square regression analysis, showed one binding of K_D 4.3 ± 0.4 nM (± s.e. mean, n = 11) with a B_{max} of 3.75 ± 0.2 pmol/g and a second site with a K_D of 38.2 ± 0.8 nM (± s.e. mean, n = 8) with a B_{max} of 12.1 ± 1.1 pmol/g. A representative Scatchard plot from two experiments is shown in Figure 3. Analysis of the saturation data by the Hill plot (Bennett, 1978), using the data for the high affinity binding site, showed that there was no cooperativity in the binding observation (see Figure 4). The line of best fit (estimated by the method of least squares) was a straight line of gradient 0.95, thus suggesting that there are no cooperative site interactions involved in the binding of low concentrations of [^{3}H]-muscimol to retinal membranes.

Examination of the subcellular distribution of specific [^{3}H]-muscimol binding showed that binding, in terms of specific activity, was greater in the P_1 fraction enriched with photoreceptor terminals (see Table 1). In these experiments, the various fractions of the retina were prepared from fresh tissue and then frozen before being assayed. The P_2 fraction which contains synaptosomes from conventional nerve-endings contained substantially fewer binding sites for [^{3}H]-muscimol than the P_1 fraction.

The substrate specificity of [^{3}H]-muscimol binding to retinal membranes was similar to that previously found in membranes derived from brain tissue (Table 2, Figure 5). 3-Aminopropane-sulphonate, GABA, isoguvacine, 4,5,6,7-tetrahydroisoxazole(5,3-C)pyridine-3-d (THIP), guvacine and imidazoleacetic acid have substantially lower IC_{50} values than bicuculline, picrotoxin or β-alanine. Of a number of putative transmitter substances tested, taurine, dopamine, noradrenaline, 5-hydroxytryptamine(5-HT), histamine and acetylcholine, none competed seriously for [^{3}H]-muscimol binding sites. Nipecotic acid and 2,4-diaminobutyric acid, inhibitors of GABA neuro-

Table 1 Binding of [^{3}H]-muscimol (5 nM) to subcellular fractions of retinal membranes

Fractions	*fmol specifically bound per mg protein*	*Enrichment*
Homogenate	12 ± 4	1.0
P_1 fraction	58 ± 8	4.8
P_2 fraction	34 ± 8	2.8

Homogenate of retina was centrifuged at 150 *g* for 10 min to remove cell debris and nuclei. The resultant supernatant was then centrifuged at 800 *g* for 10 min to obtain a P_1 pellet which was shown by Redburn (1977) to be highly rich in photoreceptor cell synaptosomes. Centrifugation of the supernatant at 25,000 *g* for 12 min produced a pellet P_2 which contains synaptosomes derived from the inner plexiform layer (Redburn, 1977). The pellets which were originally prepared in 0.32 M sucrose were suspended in 0.05 M Tris-HCl before carrying out binding assays. Results are given as the mean ± s.d. for 5 separate observations.

nal uptake, like β-alanine, an inhibitor of glial GABA uptake (Johnston, 1978), had little affinity with [^{3}H]-muscimol binding sites.

Discussion

The binding characteristics of [^{3}H]-muscimol to retinal membranes are, in general, closely comparable with those previously reported for the binding of the ligand to brain membranes, as described by Beaumont *et al.* (1978), Williams & Risley (1979), Snodgrass (1978) and Wang *et al.* (1979).

The radioligand binds specifically in a saturable manner at low concentrations to two binding sites and is slightly enhanced by freezing and treatment with Triton X-100. The absence of any enhancement of [^{3}H]-muscimol binding in the presence of sodium is of significance, since it is known that approx. 100 mM sodium causes a 10 fold increase in [^{3}H]-GABA binding (Enna & Snyder, 1975; 1977). The sodium-dependent binding of GABA is thought to represent attachment of the isotope to transport sites for GABA and since muscimol is thought to have an apparently selective affinity for the GABA receptor rather than the uptake sites (Johnston, 1976; Williams & Risley, 1979), the result is not unexpected. This observation is further supported by the finding that 2,4 diaminobutyric acid and nipecotic acid, which are both inhibitors of GABA uptake (Johnston 1978) have little affinity for [^{3}H]-muscimol binding sites.

Table 2 Pharmacological characterization of specific [^{3}H]-muscimol binding to retinal membranes

Compound	IC_{50} (μM)
Muscimol	0.03 ± 0.001
3-Aminopropanesulphonic acid	0.15 ± 0.01
GABA	0.20 ± 0.01
Isoguvacine	0.8 ± 0.03
THIP	0.85 ± 0.02
Guvacine	1.0 ± 0.09
Imidazole acetic acid	1.8 ± 0.01
Bicuculline	14.0 ± 0.1
Picrotoxin	18.0 ± 0.1
2,4L-Diaminobutyric acid	30.0 ± 0.5
Nipecotic acid	30.0 ± 0.8

The following compounds had IC_{50} value over 30 μM; taurine, dopamine, noradrenaline, 5-HT, histamine and acetylcholine. Inhibition of specific [^{3}H]-muscimol binding was as described in the text using frozen tissue and the final concentration of [^{3}H]-muscimol was 5 nM. The IC_{50} values were obtained as described in the text (some examples shown in Figure 5). Results are given as the mean ± s.d. for 3 to 9 separate observations.

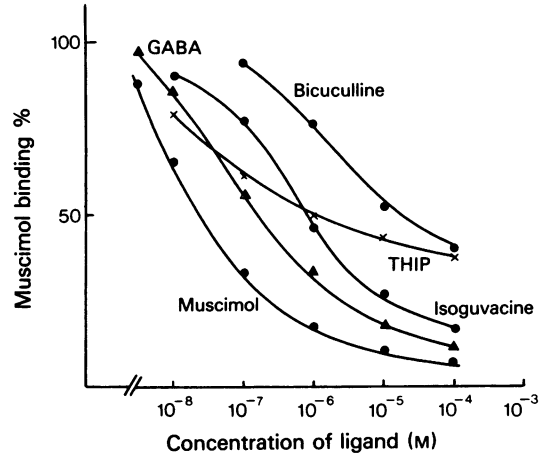

Figure 5 Binding of 5 nM [^{3}H]-muscimol to retinal membranes in the presence of various substances. Of the substances tested (see Table 2) the most potent inhibitors of [^{3}H]-muscimol binding were muscimol, GABA, isoguvacine, THIP and bicuculline, as shown above. Each point is the mean value of 6 to 9 separate determinations.

From the kinetic study of [^{3}H]-muscimol binding, it is reasonable to assume that two different binding sites occur in retinal tissue. This is supported by the Hill constant (gradient 0.95), which strongly suggests that there is no cooperative interaction associated with the high affinity binding site. The affinity constants, 4.3 and 38.2 nM, in the retina, are similar to those found in brain tissue (Beaumont *et al.*, 1978: Williams & Risley, 1979; Wang, Salvaterra & Roberts, 1979).

The substrate specificity of [^{3}H]-muscimol binding to retinal tissue provides strong evidence that the substance is highly specific for the GABA receptor. Only those substances which are known to interact neurophysiologically with the GABA receptor, e.g. 3-aminopropanesulphonic acid, have an appreciable affinity with [^{3}H]-muscimol binding sites. As would be expected, muscimol analogues, i.e. isoguvacine, THIP and guvacine, all have a high affinity for the [^{3}H]-muscimol binding sites although the nature of the displacement curves, especially for THIP (see Figure 5), suggests that the analogues do not behave in precisely the same way. The observation that the putative transmitter substances, e.g. dopamine, noradrenaline, histamine and 5-HT show little competition for the binding sites, strongly suggests that muscimol mediates via GABA and no other neurotransmitter receptors. An earlier study has additionally shown that in bovine retina there is no relationship between GABA and benzodiazepine binding sites (Osborne, 1979) which is consistent with this conclusion.

The demonstration that [^{3}H]-muscimol binding sites (assumed GABA receptor sites) have a non-uniform distribution and are primarily associated with the P_1 fraction enriched with photoreceptor terminals is in general agreement with the finding of Redburn *et al.* (1979). These authors used [^{3}H]-GABA as the ligand and showed that specific binding associated with this fraction was 78 times greater than with homogenates (Redburn *et al.*, 1979). Such a high percentage of enrichment was not observed in this study, although the results clearly show that the fraction enriched with photoreceptor synaptosomes have more binding sites for [^{3}H]-muscimol than the P_2 fraction which contains synaptosomes from conventional nerve endings. The discrepancy between the present results and those of Redburn *et al.* (1979) is difficult to understand. The exact nature of the P_1 and P_2 fraction was not analysed by electron microscopy; it is assumed that the constitutions of the two fractions are the same as those analysed by Redburn *et al.* (1979), because their fractionation procedure was used (Redburn 1977).

The slight enrichment of GABA receptors associated with photoreceptor synaptosome preparations is surprising, as the photoreceptor neurotransmitters are excitatory in nature (Dowling & Ripps, 1973), whereas GABA is an inhibitory transmitter. Since autoradiographic studies have demonstrated a high affinity uptake for GABA into horizontal cells in goldfish (Lam & Steinman, 1971), the suggestion has been made (Redburn *et al.*, 1979) that there may be GABAergic elements derived from the horizontal cell associated with the photoreceptor synaptosomes, which form the 'triad configuration of the photoreceptor cell'. The GABA receptors would, therefore, be associated with photoreceptor and/or bipolar membranes. Even if this is true, it still remains to be investigated why a significantly higher percentage of GABA receptors exists in the photoreceptor synaptosomes than in the P_2 fractions, which contain synaptosomes derived from the inner plexiform layer, where the evidence for GABA acting at the level of the amacrine neurones is persuasive (see Graham, 1974). Moreover, there is no evidence to suggest that horizontal cells in the bovine retina can specifically take up GABA or utilize it.

In conclusion, the present study substantiates the use of [^{3}H]-muscimol as a label for synaptic GABA receptors, confirming and extending the observations of others using brain tissue (Beaumont *et al.*, 1978; Snodgrass, 1978; Williams & Risley, 1979; Wang *et al.*, 1979). It also corroborates the observation made by Redburn *et al.* (1979) that subcellular fractions enriched with photoreceptor terminals contain more GABA receptors per mg tissue than fractions containing conventional synaptosomes. The presented kinetic analysis of [^{3}H]-muscimol binding should be useful for further pharmacological characterization of the GABA recognition sites in the retina. However, caution is required, since the two binding sites for [^{3}H]-muscimol have been characterized in this study on tissues untreated by Triton X-100 whilst Enna & Snyder (1976) who showed two binding sites for [^{3}H]-GABA to retinal membranes treated the tissues with Triton X-100, It is known that membranes untreated with Triton X-100 only show a single binding site for [^{3}H]-GABA (Enna & Snyder, 1976; 1977). The possibility therefore exists that muscimol may interact with other untested neurotransmitters as well as with the GABAergic system.

I would like to thank Mrs Joy Shirley for expert technical assistance and Dr James Crabbe for helping me in the statistical analysis. I am also indebted to Dr Povl Krogsgaerd-Larsen for providing me with THIP (4,5,6,7-tetrahydroisoxazole (5,3-C) pyridine-3-d), isoguvacine and guvacine.

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