MOLECULAR SIZES OF BENZODIAZEPINE RECEPTORS AND THE INTERACTING GABA RECEPTORS IN THE MEMBRANE ARE IDENTICAL

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1. Introduction

The binding sites for benzodiazepine tranquillising drugs in brain membranes are known to interact with a separate set of binding sites for γ-aminobutyric acid (GABA). Evidence for this is both pharmacological [1–3] and from binding studies [4–10] which show that GABA and certain GABA agonists enhance the binding of benzodiazepines. Support for the idea that the two sites are directly associated in one component or tightly-bound complex has come from the constancy of this linked binding in the brain at all ages from embryo to adult [11]; furthermore, either GABA-ergic or benzodiazepine ligands protect, at distinct sites, both types of binding from heat inactivation [9,10].

On solubilisation of the benzodiazepine-binding component [12], it has been found that one set of GABA-binding sites is present with it (L.-R. C., E. A. B., unpublished). However, this could arise from association in the detergent media needed, and does not prove that they are in one complex in the membrane. We have now addressed this problem by progressive inactivation of binding sites by electron bombardment of brain membranes, which can determine in a quantitative manner whether different sites are on the same molecular target. We also determine thus the size of the macromolecular structure in the membrane which carries these two types of binding site.

2. Materials and methods

Cerebral cortex was removed from 2-year-old cattle 10 min after slaughter, frozen on solid CO_2 and stored at −80°C. It was thawed, homogenised and the P_2 (lysed synaptic membrane) fraction was prepared as in [12]. This pellet was frozen at −20°C, thawed and homogenised in 32 vol. 0.05 M Tris–citrate buffer (pH 7.1)/0.05% Triton X-100 [13] and held at 20°C for 30 min. After centrifugation (48 000 × *g*, 4°C, 20 min) the pellet was washed 3 times with 32 vol. Tris–citrate buffer without Triton and once with distilled water, each time with homogenisation and centrifugation as before. These treatments remove all endogeneous GABA and hence maximise [13] specific, high-affinity [^{3}H]muscimol binding. Another sample was prepared similarly but using 0.01% Triton X-100 at 4°C in place of 0.05% Triton at 20°C, and then freeze–thawed and given an additional wash in the Tris–citrate buffer; this gave no difference in the K_d-values or irradiation behaviour to the first set of samples. Hence, the effects found do not depend upon a particular detergent treatment. Protease inhibitors [12] were present at all stages up to, but not beyond, the final wash.

The washed pellet in each case was re-homogenised in distilled water containing two enzyme markers (see below); aliquots were lyophilised in thick-wall Pyrex tubes and sealed in vacuo. These were irradiated in the Cambridge linear accelerator [14]; after various (metered) incident radiation doses at 20–30°C, the vacuum was broken and the membranes re-homogenised at 4°C in Tris–citrate buffer. Appropriate aliquots were taken in duplicate from each sample for measuring binding of each ligand, for assay of each enzyme marker and for protein determination. Binding assays used [*N-methyl*-^{3}H]flunitrazepam (75 Ci/mmol) or [*methylamine*-^{3}H]muscimol (9.5 Ci/mmol), both from the Radiochemical Centre [1]. In the standard assays 40 nM [^{3}H]flunitrazepam or 50 nM [^{3}H]mus-

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cimol was used to follow the binding loss at the high affinity sites. Scatchard plots [12] of a range of binding data for the non-irradiated and the partly-inactivated membranes confirmed that those fixed concentrations represented saturation of binding to one class of high-affinity sites. In all assays non-specific binding was subtracted, using controls with 1 μM clonazepam or 100 μM GABA, respectively, added. The specific binding was always expressed per mg protein as measured in the same sample, to correct for any variation between membrane samples.

The internal enzyme markers were alcohol dehydrogenase (ADH) from yeast (2 units/tube) and *Escherichia coli* β-galactosidase (25 units/tube), the purest grades from Sigma. Other methods were as in [12].

3. Results

The binding of [^{3}H]flunitrazepam to bovine cerebral cortex membranes (prepared in the presence of protease inhibitors), after freeze–thawing and extensive washing (see section 2), when represented as a Scatchard plot as for rat cortex [12], showed a single class of binding sites, with K_d = 5.5 nM and saturation binding of 1.3 pmol/mg protein. These values are of the same order as found for rat cortex [12] and agree with a report [9] for bovine cortex. The binding of muscimol has been shown [13,15,16] to correspond to GABA binding to its synaptic receptor sites, and was used here to show the latter. Freeze-thawed, thoroughly-washed membranes (see section 2) were used to remove endogenous inhibitors, including GABA [8,17]. When [^{3}H]muscimol was used, similar binding analysis showed that up to 50 nM ligand the binding could be represented by one set of high-affinity sites, binding at their saturation 1.2 pmol/mg protein with K_d = 5.8 nM. This, also, agrees fairly well with the report [9] on bovine brain membranes (prepared differently). These bindings were abolished by the presence of unlabelled clonazepam (10^{-6} M) or GABA (10^{-4} M), respectively; i.e., they are specific.

When the membranes were rapidly frozen in liquid N_2 and freeze-dried, no loss of binding of either ligand occurred, the same binding parameters being obtained upon re-hydration. Samples of these membranes were irradiated to various total dosages and the decline in the numbers of binding sites present was followed (fig.1). It was clearly seen that the rate of loss of the benzodiazepine binding sites was equal to that of the

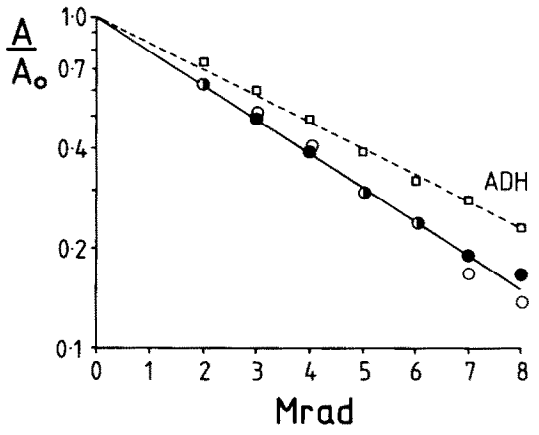

Fig.1. Radiation dose-dependence of [^{3}H]flunitrazepam binding (○) and of high-affinity [^{3}H]muscimol binding (●) in a given sample of bovine cortex membranes. (Where the two values coincided, half-shading is shown.) Also shown (□) is the enzymic activity of yeast alcohol dehydrogenase (ADH) irradiated in the same specimen, as one of the molecular mass markers. A/A_0 is the activity (binding or enzymic) as a fraction of the initial activity (i.e., 1.0 at 0 Mrad), on a logarithmic scale. The lines were fitted by least squares. The slope of the binding activity plot (the dose-dependence) divided by the slope of the ADH plot is the inactivation ratio for ADH as the reference, as used in table 1. The steeper the dose-dependence slope, the larger the target molecular size.

muscimol binding sites. Repeats of these experiments on 3 other brain cortex samples gave in every case an identical plot for the two properties. Binding of these ligands elsewhere (i.e., non-specific) decayed much more slowly than the specific binding of each, and the bindings of other types of ligand to these membranes also showed quite different dose-dependences (unpublished). It is noteworthy that both types of specific binding site here lose activity in a single exponential process, in line with the single class of each identified with the high-affinity binding.

By well-established target theory, the actual dose-dependence is related to the molecular size of the target unit required for maintaining the activity in question (review [18]). We have used the rate of loss of the binding site relative to that of enzymes of known size irradiated in the same sample, as the most accurate measure of the target size, avoiding other assumptions which may not apply to proteins in biological membranes. Using yeast alcohol dehydrogenase (M_r 149 000) and *E. coli* β-galactosidase (M_r 464 000 [19]) as these standards, their inactivation dose-dependence, relative to that of the benzodiazepine binding

Table 1
Molecular masses of targets for radiation inactivation of specific binding sites

Binding site	K_d (nM)[a]	Reference protein[b]	Inactivation ratio[b]	Molecular mass[c]
[^{3}H]Flunitrazepam	5.5	β-Galactosidase	0.542	216 000 ± 2500
		Yeast ADH	1.25	
[^{3}H]Muscimol	5.8	β-Galactosidase	0.547	217 000 ± 1900
		Yeast ADH	1.25	

[a] Dissociation constant of the sites monitored. This did not change significantly during the treatments

[b] The reference enzyme named was irradiated in the same specimens, and its dose-dependence (the slope of a plot as in fig.1) was measured. The ratio of the dose-dependence for the ligand binding site inactivation to the reference dose-dependence is the inactivation ratio

[c] A linear calibration plot was employed, which relates the molecular masses of 6 irradiated standard proteins to their inactivation ratios based upon either of the two reference proteins used here. (Full details of the calibration method will be presented elsewhere.) The inactivation ratio in each case here was converted thus to the corresponding target molecular weight. This is given as the mean (±SEM) for 4 expt of the type illustrated in fig.1

(fig.1) during the same irradiation, yielded by comparison a value for the functional size required to maintain the binding site (table 1). In all of the experiments this was the same as the value obtained in the case of the muscimol binding site (table 1), confirming the behaviour illustrated in fig.1.

4. Discussion

To progressively destroy the two types of site-benzodiazepine-binding and GABA-binding – by electron bombardment is a procedure analogous to comparing their thermal inactivations. However, it differs in that with the irradiation a discrete packet of energy is applied which will destroy totally the target molecule if it impinges on it, and hence the parameter of the dose-dependence of the radiation effect offers a quantitative and more sensitive test for the identity of two targets. By this test they are identical in the membrane. Of course, this could arise by a coincidence in the size of two different macromolecules. The latter appears to be a very improbable explanation in the present case, in the light of all of the other types of evidence (see section 1) that one site modulates binding at the other.

The other important advantage of the radiation method is that it, unlike thermal inactivation, can actually measure that target size. We obtain a value of 217 000 M_r for the weight of the component which carries both types of site. This may be either one protein, or a complex if its component proteins are tightly enough bound together in ther membrane to serve as one functional unit, which is the significance of the target size. When solubilised in detergent [12], a sedimentation coefficient of ~9.5 S in sucrose density gradient centrifugation has been found in this laboratory for both the muscimol-binding component and the associated flunitrazepam-binding component (L.-R. C., E. A. B. unpublished). Considerable inaccuracy is possible in relating the sedimentation coefficient of a membrane protein in aqueous detergent solution to its molecular mass [20]: depending upon the amount of bound detergent and the degree of molecular asymmetry, the 9.5 S value could be due to a molecular complex of 217 000 M_r, but further analysis would be needed to establish the true molecular mass in the solubilised form.

There are several classes of GABA-binding site in brain membranes, but in the cerebral cortex muscimol has been found to bind with high affinity ($K_d < 10$ nM) to the Na^+-independent class of these, apparently linked to chloride conductance, which corresponds to synaptic GABA receptors [7,13,15,16]. We monitored only this high affinity muscimol binding here, which behaves as a single class in its inactiva-

tion (fig.1). It is this class which we show as linked to the benzodiazepine-binding site. The evidence presented or reviewed does not, however, exclude that upon experimental fractionation these sites could be found on different polypeptides. It does, however, now show that in the membrane the two activities are associated in one functional structure, and this has a molecular mass of 217 000 M_r.

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