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ENHANCED BINDING OF THE CONVULSIVE LIGAND DMCM TO HIGH-ENERGY IRRADIATED BENZODIAZEPINE RECEPTORS; EVIDENCE OF COMPLEX RECEPTOR STRUCTURE

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It is generally believed that the GABA/benzodiazepine receptor complex contains binding sites not only for GABA but also for benzodiazepines and barbiturates, and coupling between these binding sites has been described (1-5). Benzodiazepines probably exert their effect by facilitating GABA-mediated neurotransmission while some convulsant β-carboline-3-carboxylates, such as β-CCM and DMCM might exert their effect by reducing GABA mediated neurotransmission (6, 7).

^{3}H-DMCM apparently binds to benzodiazepine receptors in rat brain (8). Binding of ^{3}H-DMCM has high affinity for its binding sites ($K_D \simeq 0.5$-5 nM, with curvilinear Scatchard plot); benzodiazepine receptor ligands have high affinity for ^{3}H-DMCM binding sites; and GABA reduces specific ^{3}H-DMCM binding under appropiate conditions. The present preliminary report describes an unexpected increase in ^{3}H-DMCM binding upon high-energy irradiation of rat cortical membranes.

MATERIALS AND METHODS

Materials: ^{3}H-DMCM (^{3}H-methyl 6,7-dimethoxy-4-ethyl-β-carboline-3--carboxylate 75.3 Ci/mmol) was prepared by Richard Young, NEN, Boston Mass. ^{3}H-FNM (^{3}H-flunitrazepam 79 Ci/mmol) were purchased from NEN, Boston.

Radioligand binding assays: Aliquots of the membrane suspensions were incubated at 0°C with either ^{3}H-DMCM (final concentration 0.1 nM in 2.50 ml) or ^{3}H-FNM (final concentration 1 nM in 1.00 ml) for 60 min. Bound and free radioactivity was separated by filtration through Whatman GF/C glass fibre filters and washing with 3x5 ml buffer and was measured by conventional techniques. Specific binding was obtained by subtracting from the total bound radioactivity, binding in the presence of midazolam (final concentration 1 μM). Further details in legend fig. 1.

RESULTS

Inactivation of specific ^{3}H-FNM binding to rat cortex by high-energy irradiation is shown in fig. 1. The apparently monoexponential decay suggests loss of a single binding component of homogenous size. Accurate calibration of the decay function will not be reported here, the presented absolute values of molecular weights should be regarded as approximations. The

radiation inactivation constant was determined from the slope in fig. 1, k = 0.085 $Mrad^{-1}$, which according to Kepner and Macey (9) roughly correspond to a molecular weight of the ^{3}H-FNM binding site of ca. 50,000 daltons, (MW = $6.4 \times 10^5 \cdot k$, $k=1/D_{37}$). The inactivation of ^{3}H-DMCM binding proceeds in a curvilinear fashion (fig. 1).

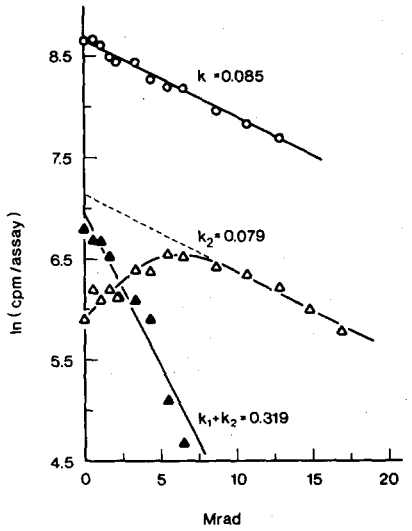
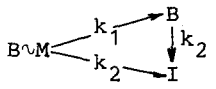


Fig. 1. Radiation inactivation of ^{3}H-FNM (o) and ^{3}H-DMCM (Δ) binding sites. The inactivation curve of B∿M (▲) was obtained by subtraction of the extrapolated linear part (---) and the experimental inactivation curve of ^{3}H-DMCM binding sites. The irradiation experiments were done on whole rat cerebral cortex homogenized in 20 vol (w/v) of ice-cold 50 mM tris-citrate, pH 7.1. Aliquots of 400 µl of membrane suspension were rapidly frozen in sealed glass tubes at ca. -20°C. The samples were exposed to high energy electrons using the 10 MeV linear accelerator at Risø, Denmark. The dose of radiation was determined using calibrated thermo dosimeters (water). The samples were cooled (ca. -10°C) during irradiation which was delivered in runs of 0.5-2 Mrad. In between runs, samples were cooled to -15°C for at least 2 min to insure that they remained completely frozen during the whole irradiation process. After storage for 1-2 days at -20°C the samples were thawed homogenized in 500 vol (w/v) of 50 mM tris-citrate pH 7.1. Radioligand binding assays were done as described.

The apparently monoexponential component of the curve had a slope similar to the slope of the ^{3}H-FNM binding inactivation curve, and consequently the same molecular weight. The curvilinear nature of the inactivation could be explained assuming an inactivation following the scheme:



$$(B\sim M)_D = (B\sim M)_0 \cdot e^{-(k_1+k_2)D} \text{ and}$$

$$B_D = (B_0 + (B\sim M)_0) \cdot e^{-k_2 D} - (B\sim M)_0 \cdot e^{-(k_1+k_2)D}$$

where B∿M is the ^{3}H-DMCM binding site (B) attached to a high molecular weight molecular species (M) which reduced binding to B. ^{3}H-DMCM does not bind to I, the inactivated binding site. The constants k_1 and k_2 are the radiation inactivation constants (in $Mrad^{-1}$) for M and B, respectively. Solving the differential equations of the reaction scheme, shown above, and assuming that the dissociation constant of DMCM binding to B, K_{DB}, is ca. ten fold lower than K_{DBM} the dissociation constant of DMCM binding to B∿M, and that ^{3}H-DMCM binding was studied at a concentration well below K_{DB}, the curvilinear inactivation curve of ^{3}H-DMCM binding can be resolved into two linear forms by substracting the experimental from the extrapolated

linear component. The high molecular weight component had a radiation inactivation constant, k_1 = 0.240 $Mrad^{-1}$, corresponding to a molecular weight of approximately 150,000 daltons. Scatchard analyses of ^{3}H-DMCM binding to membranes after 10 Mrad irradiation showed increased contingency of the high affinity component as compared to 0 Mrad in agreement with the above conjectures (data not shown).

Irradiation in aquous media, also in the frozen state, is known to cause radical formation, which in turn may affect inactivation, making molecular weight determination inaccurate. However, radiation inactivation in lyophilized membranes at -10°C, where free radical formation is low, yielded similar curvilinear inactivation curves for ^{3}H-DMCM binding sites.

DISCUSSION

The results of the present study show that the molecular target size of the binding protein for ^{3}H-FNM and the convulsive β-carboline, DMCM, is almost the same when irradiated under identical conditions, suggesting that the two compounds interact with the same binding protein. This is in accordance with the binding properties of ^{3}H-DMCM as compared to that of ^{3}H-FNM (8).

The curvilinear radiation inactivation curve found for the ^{3}H-DMCM binding site suggests that binding is increased as a consequence of destroying a high molecular weight component as previously suggested also for insulin binding sites (10). Subtracting the curvilinear part from the extrapolated linear part we find an apparently monoexponential rapid decay for the regulating component which suggest a molecular weight of ca. 150,000 daltons. The nature of the high molecular weight regulatory structure is unknown; for example it could be a new protein or a complex structure of known proteins. Previous molecular weight determination have revealed molecular weights of benzodiazepine receptors of ca. 50,000, 100,000 or 200,000 daltons depending on the conditions for investigation (3, 11-14). Our results are compatible with the occurence of benzodiazepine receptors as a tetrameric subunit complex, B could represent one binding protein and M would correspond to the three others. If the tetrameric nature of the complex is split by hitting any one of the subunits the affinity for ^{3}H-DMCM of the remaining units might increase.

Irradiation inactivation is not the only way ^{3}H-DMCM binding can be enhanced. Mild treatment of brain membranes with 0.05% Triton X-100 enhances the affinity of ^{3}H-DMCM binding (unpublished), treatment with 0.1 mM Ag^+ enhance ^{3}H-DMCM binding ca. 8 fold (8) and exposure of brain membranes to UV treatment in the presence of flunitrazepam likewise enhance ^{3}H-DMCM binding substantially (8). Again it is not known whether these enhancements are related to the increased binding after irradiation described above.

Many lines of evidence suggest that the β-carbolines and the benzodiazepines binds to a common site or at least overlapping recognition sites. However, binding of these groups of ligands does not occur in an identical manner (7, 15, 16). ^{3}H-DMCM binding exhibits curvilinear Scatchard plot indicative of heterogeneity of binding sites (8). Enhanced binding of ^{3}H-DMCM after irradiation or other treatments, however, can not be explained only on basis of selective destruction or solubilisation of distinct binding sites, but requires also a regulatory function, which from the irradiation experiments seems to involve a high molecular weight structure. Apparent binding heterogeneity of ^{3}H-DMCM binding observed in Scatchard analyses might represent the low affinity B↷M structure ($K_{DBM} \approx 5$ nM) and the high affinity B-structure ($K_{DB} \approx 0.7$ nM).

In summary, the present work demonstrates a similar molecular weight of FNM and high affinity DMCM binding sites, and an increased binding of the convulsive β-carboline DMCM upon irradiation of cortical membranes.

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REFERENCES

1. Tallmann, J.F., Thomas, J.W. and Gallager, D.W. Nature 274 383 (1978).
2. Braestrup, C., Nielsen, M., Krogsgaard-Larsen, P. and Falch, E. Nature 280 331 (1979).
3. Sieghart, W. and Karobath, M. Nature 286 285 (1980).
4. Leeb-Lundberg, F., Snowman, A. and Olsen, R.W. Proc. Natl. Acad. Sci. USA 77 7468 (1980).
5. Ticku, M.K. Biochem. Pharmacol. 30 1573 (1981).
6. Braestrup, C. and Nielsen, M. Nature 294 472 (1981).
7. Braestrup, C., Schmiechen, R., Neef, G., Nielsen, M. and Petersen, E.N. Science 216 1241 (1982).
8. Braestrup, C., Nielsen, M. and Honoré, T. J. Neurochem. (Submitted).
9. Kepner, G.R. and Macey, R.I. Biochim. Biophys. Acta 163 188 (1968).
10. Harmon, J.T., Kahn, C.R., Kempner, E.S. and Schlegel, W. J. Biol. Chem. 255 3412 (1980).
11. Möhler, H., Battersby, M.K. and Richards, J.G. Proc. Natl. Acad. Sci. USA 77 1666 (1980).
12. Tallman, J.F., Mallorga, P., Thomas, J.W. and Gallager, D.W. in GABA and Benzodiazepine Receptors, Eds. E. Costa, Di Chiara, G. and Gessa, G.L. Raven Press, New York, 9 (1981).
13. Braestrup, C., Nielsen, M., Skovbjerg, H. and Gredal, O. in GABA and Benzodiazepine Receptors, Eds. E. Costa, Di Chiara, G. and Gessa, G.L. Raven Press, New York, 147 (1981).
14. Doble, A. and Iversen, L.L. Nature 295 522 (1982).
15. Nielsen, M., Schou, H., Braestrup, C. J. Neurochem. 36 276 (1981).
16. Hirsch, J.D. Pharmacol. Biochem. Behav. 16 245 (1982).