Vol. 126, No. 1, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 16, 1985 Pages 227-232

MOLECULAR SIZE OF THE HIGH-AFFINITY GLUTAMATE-BINDING SITE ON SYNAPTIC MEMBRANES FROM RAT BRAIN

Marie E. Bardsley* and Peter J. Roberts

Department of Physiology & Pharmacology, University of Southampton, Medical & Biological Sciences Building, Bassett Crescent East, Southampton SO9 3TU, U.K.

Received November 21, 1984

We have used radiation inactivation as a means of determining the molecular size of the high-affinity glutamate-binding site on rat brain synaptic membranes. The molecular size was 75,000 ± 15,000 in the absence of glutamate and 263,000 ± 34,000 in the presence of glutamate. These data may be interpreted as suggesting that the high-affinity glutamate-binding site is comprised of a number of subunits. The minimum sub-unit size detected by this method was 75,000 ± 15,000.

L-glutamate is a potent excitant of neuronal cells and is probably the dominant excitatory neurotransmitter in the CNS (1,2). Electrophysiological (3,4) and radio-labelled ligand binding studies (5-7) indicate that there may be at least three distinct classes of receptor at the functional level. However, a single glutamate-binding protein of very small molecular weight has been solubilised and purified from rat and bovine brain (8-10). In this study we have applied the technique of radiation inactivation to gain information on the molecular size of the high-affinity glutamate-binding site on synaptic membranes. The technique of radiation inactivation has been widely employed to determine the molecular size of enzymes (11) and receptors (12-14). It is the only method which can be used to investigate the molecular size(s) of membrane-bound proteins/functions without prior solubilisation; the latter is very dependent on the availability of tight-binding probes or the presence of distinguishing receptor-associated functions. Radiation inactivation relies on the 'single-hit' hypothesis i.e. if one electron hits the protein, its function is completely destroyed.

* To whom correspondence should be addressed.

0006-291X/85 $1.50

It therefore follows that the remaining activity will decrease as a simple exponential function of dose

$$X = X_o e^{-Kr}$$

where X_o and X are the concentrations of target molecules before and after receiving a dose of radiation r. K is a constant proportional to the molecular mass of the molecule or target. An empirical equation relating dose to molecular size has been derived (15)

$$Mr = \frac{6.4 \times 10^5}{D_{37}}$$

where Mr is the molecular size and D_{37} the dose of radiation at which 37% of the activity remains. The equation is widely used and its validity borne out by the number of cytosolic and membrane proteins for which this procedure has either given the whole or partial molecular weight (11). It is suggested that the molecular size determined by radiation inactivation represents the 'functional unit' rather than the 'structural object' (11,16). Further information on the theory and empirical justifications of this method can be found in references 17, 18.

Methods

Whole brains from adult, male Wistar rats were excised and homogenised in 0.32 M sucrose buffered with 5 mM Hepes, pH 7.4. Following a slow spin at 760 x g for 15 min, the supernatant was spun at 27,000 x g for 15 min. The pellet was resuspended in 0.32 M sucrose, layered onto a sucrose density gradient comprising 0.8 M and 1.2 M sucrose buffered with 5 mM Hepes pH 7.4 and then centrifuged at 48,400 x g for 90 min. The synaptosomes thus prepared were lysed in 5 mM Hepes pH 7.4 and the membranes spun down at 48,400 x g for 10 min. The membranes were washed a further three times in 5 mM Hepes pH 7.4 then finally resuspended in a small volume of buffer. Glutamate was added to half of this volume at a concentration of 50 μM. Small samples (approx. 0.3 ml; 1.5 mg protein; 0.9 g original wet weight) were then rapidly frozen in liquid nitrogen and freeze-dried at 0.03 millibar for 24 h.

The samples were maintained under vacuum during irradiation in the beam of 15-MeV electrons from a Phillips MEL SL 75-20 linear accelerator at the Department of Radiotherapeutics, New Addenbrooke's Hospital, Cambridge, England. The tubes containing the samples were pre-cooled and kept cool with a stream of dry-ice cooled air. The samples were not exposed to more than 5 MRad without a period of cooling. These conditions together with the use of lyophilised membranes are designed to eliminate inactivation by free radicals (15,18). The dose of radiation was calibrated by:-

(a) Using the Perspex optical density method of Berry & Marshall (19).

(b) The dose rate was 2 MRad/min and the dose was determined by an integrated current flow to earth from the aluminium support block.

(c) Using acetylcholinesterase as an internal standard.

The binding of [^{3}H]-glutamate to the freeze-dried irradiated membranes was measured after washing 4 times in 5 mM Hepes, pH 7.4. Specific, high-affinity binding was defined as the amount of label that could be displaced by 1 mM cold glutamate.

The binding of [^{3}H]-glutamate to lyophilised membranes showed a single, saturable binding site; the Kd was the same as that reported for fresh membranes (359 + 133 nM) but the Bmax increased from 117 + 17 pmol/mg to 277 + 48 pmol/mg (20). This appears to be due to the release of an inhibitor on lyophilisation; this inhibitor is removed by the washing procedure outlined above.

Acetylcholinesterase was assayed by the method of Ellman *et al* (21).

Protein was assayed by the method of Lowry *et al* (22) after precipitation with trichloroacetic acid.

Results

The data presented here show the results from two irradiations per dose, three samples per dose and condition (plus or minus glutamate) and triplicate assays for each sample. These data were fitted to a simple exponential by derivative-free non-linear regression. This number of irradiations and samples minimises the need for other methods of standardisation or calibration.

The synaptic membranes which were freeze-dried in the absence of glutamate gave a molecular size of 75,000 ± 15,000 (Fig. 1a); those freeze-dried in the presence of glutamate gave a molecular size of 263,000 ± 34,000 (Fig. 1a). For clarity, the figure shows the log of the remaining activity versus the radiation dose; the line of best-fit was calculated by non-linear regression. The increase in molecular size seen in the presence of glutamate suggests a possible 'collision coupling' (cf. 23, 24) of receptor sub-units (effector molecules e.g. sodium channels) to the ligand recognition site. Although it is possible to calculate sub-unit numbers, such interpretations are problematic and should be viewed with caution.

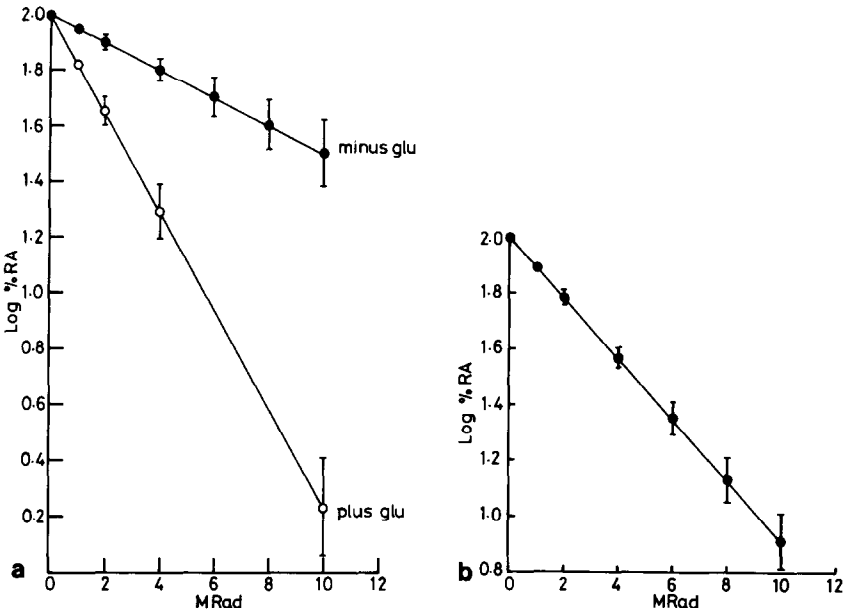

Figure 1a shows the decrease in log % remaining activity of glutamate binding to lyophilised rat brain synaptic membranes versus the dose of radiation in megarads. The filled circles show the points obtained in the presence of glutamate and the open circles show the points obtained in the absence of glutamate. Figure 1b shows the decrease in log % remaining activity of acetylcholinesterase in the same preparation of membranes versus the dose of radiation in megarads. The points were calculated from samples with and without added glutamate. Each point was calculated by derivative-free non-linear regression from the data; the error bars denote the standard deviation of the data from the predicted point.

Since, from both electrophysiological and ligand-binding data, there would seem to be a number of excitatory amino acid receptors, for which glutamate is a substrate, our data would tend to suggest either (a) only one receptor sub-type was measured by this method (e.g. the aminophosphono-butyrate receptor/binding site, see reference 7) or (b) the receptor subtypes have similar molecular sizes. Further experimentation with other ligands may help to resolve this question.

Our data suggest that if the glutamate-binding protein isolated by Michaelis (8-10; molecular weight 14,300) does represent the high-affinity glutamate binding site on synaptic membranes then (a) it is only a fragment of the whole, (b) it exists as aggregates within the membrane, (c) the method described here is measuring a protein aggregate.

The molecular size of acetylcholinesterase was 161,000 ± 14,000 (Fig.1b). This suggests that the G2 form of the enzyme predominates in

lyophilised synaptic membranes from rat brain (for review see 25). The size of G1 (the catalytic sub-unit, as determined by radiation inactivation, is 75,000 (26) ; our results were consistently twice this value and unaltered by the presence or absence of glutamate. Hence although it is conceivable that our molecular sizes are larger than the real values, the relative alteration in size seen in the presence of glutamate remains.

In conclusion the data presented here provide the first direct information on the molecular size of a high-affinity glutamate-binding site on synaptic membranes and its increase in the presence of glutamate.

Acknowledgements

We are grateful to Martin Brand and Simon Hughes for their help. This work was supported by a project grant from the Wellcome Trust.

References

1. Roberts, P.J., Storm-Mathisen, J. and Johnston, G.A.R. (1981) Glutamate: Transmitter in the Central Nervous System, Wiley, Chichester, U.K.
2. Fagg, G.E. and Foster, A.C. (1983) Neuroscience 9 , 701-709.
3. Watkins, J.C. (1981) Glutamate: Transmitter in the Central Nervous System, pp 1-24, Wiley, Chichester, U.K.
4. Watkins, J.C., Davies, J., Evans, R.H., Francis, A.A. and Jones, A.W. (1981) Psychopharmacol. 27 , 263-273.
5. Fagg,, G.E., Foster, A.C., Mena, E.E. and Cotman, C.W. (1982) J. Neuroscience 2 , 958-964.
6. Roberts, P.J. and Butcher, S.P. (1983) The metabolic relationship of glutamine, glutamate and GABA, pp. 517-536, A. Liss, New York.
7. Foster, A.C. and Fagg, G.E. (1984) Brain Res. Rev. 7 , 103-164.
8. Michaelis, E.K., Michaelis, M.L., Chang, H.H., Grubb, R.D. and Kuonen, D.R. (1981) Mol. Cell. Biochem. 38 , 163-179.
9. Michaelis, E.K., Michaelis, M.L., Stormann, T.H., Chittenden, W.L. and Grubbs, R.D. (1983). J. Neurochem. 40 , 1742-1753.
10. Michaelis, E.K., Chittenden, W.L., Johnson, B.E., Galton, N. and Decedue, C. (1984) J. Neurochem. 42 , 397-411.
11. Kempner, E.S. and Schlegel, W. (1979) Anal. Biochem. 92 , 2-10.
12. Lo, M.M.S., Barnard, E.A. and Dolly, J.O. (1982) Biochemistry 21 , 2210-2217.
13. Chang, L-R., Barnard, E.A., Lo, M.M.S. and Dolly, J.O. (1981) FEBS Lett. 126 , 309-311.
14. Doble, A. and Iversen, L.L. (1982) Nature 295 , 522-523.
15. Kepner, G.R. and Macey, R.I. (1963) Biochem. Biophys. Acta 163 , 188-203.
16. Ottolenghi, P. and Ellory, J.C. (1983) J. Biol. Chem. 288 , 14895-14907.
17. Pollard, E.C. (1959) Biophysical Science - A Study Program, pp. 273-278, Wiley & Sons, New York.
18. Okada, S. (1970) Radiation Research, pp. 1-76, Academic Press, London.
19. Berry, R.J. and Marshall, C.H. (1969) Phys. Med. Biol. 14 , 585-596.
20. Sharif, N. (1981) Ph.D. thesis, University of Southampton, U.K.

21. Ellman, G.L., Courtney, D., Andres, V.Jr. and Featherstone, R.M. (1961) Biochem. Pharmacol. 7 , 88-95.
22. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193 , 265-275.
23. Houslay, M.D. (1981) Advances in Cyclic Nucleotide Research 14 , pp. 111-119. Raven Press, New York.
24. Houslay, M.D., Ellory, J.C., Smith, G.A., Hesketh, T.R., Stein, J.M., Warren, G.B. and Metcalfe, J.C. (1977) Biochem. Biophys. Acta 467 , 208-219.
25. Massoulié, J. and Bon, S. (1982) Ann. Rev. Neurosci. 5 , 57-106.
26. Levinson, S.R. and Ellory, J.C. (1974) Biochem. J. 137 , 123-125.