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THE GLUTAMATE RECEPTOR-LIKE PROTEIN OF BRAIN SYNAPTIC MEMBRANES IS A METALLOPROTEIN

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SUMMARY: The glutamate binding glycoprotein from rat brain synaptic plasma membranes was found to be quite sensitive to inhibition by sodium azide (NaN_3). The glutamate binding activity of the purified glycoprotein was decreased to 50% in the presence of 0.33 mM NaN_3. Similar concentrations of NaN_3 also caused 50% inhibition of glutamate binding to the synaptic membranes. Analysis of the purified binding protein for its content of iron and sulfur revealed the presence of 1.95 g atom Fe and 2.19 g atom S per mole of 14,000 M.W. protein. On the basis of changes in the difference spectra of this protein it is believed that both the azide effect and the glutamate binding are taking place at a site which contains the Fe_2-S_2 center.

Isolated plasma membranes from the synaptic region of rat brain neurons are known to contain a high affinity, stereoselective, L-glutamate binding protein (GBP) which has many of the expected characteristics of the receptor for this neuroexcitatory amino acid (1,2). On the basis of this protein's high affinity for L-glutamic acid and its strong interaction with the plant lectin concavalin A (1), we have developed a purification scheme for GBP which involves a two step affinity chromatographic separation through L-glutamate-loaded glass fiber and concanavalin A sepharose (2). This procedure has allowed us to obtain a nearly homogeneous preparation of this membrane glycoprotein which we have subsequently employed in studies of its chemical, physical, and pharmacological properties. This protein lacks any glutamate metabolizing activity and differs in many of its characteristics from this amino acid's uptake carrier at the synaptic membranes (2).

During the conduct of these experiments the observation was made that the addition of sodium azide (NaN_3) as an antibacterial agent caused a complete loss

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of this protein's binding activity. This observation has led us to examine the possible mechanism of action of NaN_3, especially with respect to the existence of a metal-containing center, in particular, an iron center at the ligand-binding site of this protein. The reason for such an exploration is based on the knowledge that azide ions interact rather strongly with the iron ions of various metalloproteins, and frequently cause inhibition of the activity of these proteins (e.g., 3,4,5).

MATERIALS AND METHODS

Tissue Preparation and Protein Purification: The procedures employed for the preparation of the synaptic plasma membranes and for the purification of the GBP are identical to those previously described (1,2). The glutamate binding fractions obtained following elution from the concanavalin A sepharose column were extensively dialyzed against a 10 mM potassium phosphate buffer, pH 7.45, or against the same buffer which contained 0.5% v/v Triton X-100. The dialyzed fractions were finally concentrated by ultrafiltration and stored at 4°C in the absence of any additives.

Binding and Protein Assays: L-Glutamate binding activity of the purified GBP was measured by a Millipore filtration assay employing L-[^{3}H]-glutamic acid (40 Ci/mmole, New England Nuclear Corp.) as previously described (2). All incubations for ligand binding assays were conducted at room temperature for 25 min, a time period which was shown to be more than sufficient for the system to reach complete equilibrium. Binding of L-[^{3}H]-glutamic acid to rat brain synaptic membranes was measured by a microfuge centrifugation assay. In summary, 5 µl of the synaptic membrane preparation (1.5 mg protein/ml) was added to a 95 µl mixture of 10 mM potassium phosphate buffer, 0.1 mM p-chloromercuriphenylsulfonate (PCMPS) and L-[^{3}H]-glutamic acid. The binding equilibrium was allowed to take place in micrafuge tubes at room temperature for 25 min. PCMPS is routinely added since it blocks 80% of L-glutamate uptake but does not affect either receptor function (6) or glutamate binding (2). Non-specific L-glutamate binding was estimated by simultaneously incubating synaptic membranes with L-[^{3}H]-glutamate in the presence of an excess of non-radioactively labelled L-glutamate concentration (10^{-4} M). All reactions were stopped by the rapid addition of 0.2 ml of ice-cold phosphate buffer, transfer of the tubes to ice for 30 sec, and finally centrifugation of the tubes, in a Beckman Microfuge for 1.5 min. Following aspiration of the supernatant, the membrane pellets were disolved in 50 µl of 1N NaOH and 1 ml of 2-ethoxyethanol, and the radioactivity was measured in a 2-ethoxyethanol: toluene (0.2: 0.8 v/v) based scintillation fluid. Non-specific [^{3}H]-glutamate binding averaged about 4-6% of the total and was subtracted from all samples. Protein concentration of all preparations was measured by the Lowry procedure (7).

Determination of Fe and Labile S: The content of iron in purified GBP was determined by the method of DeBogart and Beinert (8) and the amount of labile sulfur by the method of Lowenberg and colleagues (9).

RESULTS AND DISCUSSION

The observation that storage of the purified GBP in the presence of 3.3 mM NaN_3 led to complete loss of the protein's glutamate binding activity was ex-

plored further by determining the effects of various concentrations of NaN_3 on the glutamic acid binding activity of GBP. As is shown in Fig. 1, the preincubation of the protein for 5 min with a number of concentrations of NaN_3 resulted in a concentration-dependent blockade of the L-glutamic acid binding activity. The concentration of NaN_3 causing 50% inhibition of binding (IC_{50}) was 0.33 mM (Fig. 1), and NaN_3 at 3.3 mM concentration completely inhibited the binding interactions between L-glutamic acid and GBP. It was considered possible that these effects of NaN_3 on the purified protein may be a manifestation of the sensitivity of GBP to this agent only when the protein is in its purified state. This possibility was explored by determining the effects of NaN_3 on the stereoselective, high affinity binding of L-glutamic acid to the synaptic membranes. The data in Fig. 2 indicate that L-glutamic acid binding to the synaptic membranes is inhibited by the same concentrations of NaN_3 shown to affect binding to the purified protein. The estimated IC_{50} for NaN_3 inhibition of L-[^{3}H]-glutamic acid binding to synaptic membranes is 0.372 mM, a value quite similar to that observed for the purified GBP. It would appear, then, that both the synaptic membrane glutamate binding site and the purified protein are equally sensitive to NaN_3. This sensitivity of the glutamate binding system in either state is not due to the increase in Na+, which we have found to have no effect at these concentrations, but rather to the presence of the N_3 ion.

Azide ions are known to bind to metal ions, such as Fe, which exist at the active site of metalloproteins and to act as inhibitors of the activity of these proteins (e.g., 3,4,5,10). The effects of NaN_3 on glutamate binding are suggestive of the possibility that the GBP is also a metalloprotein, more specifically an iron containing protein. Analysis of the iron content of two different batches of purified GBP gave a value of 1.95 $\pm$ 0.18 g atoms Fe per mole of 14,000 dalton protein, which is the estimated molecular weight of the GBP obtained from SDS electrophoresis (2). Since the iron at the active site of several metalloproteins is known to exist in an iron-sulfur linkage (11), it was considered likely that a similar arrangement might exist in the case of the GBP. When the same

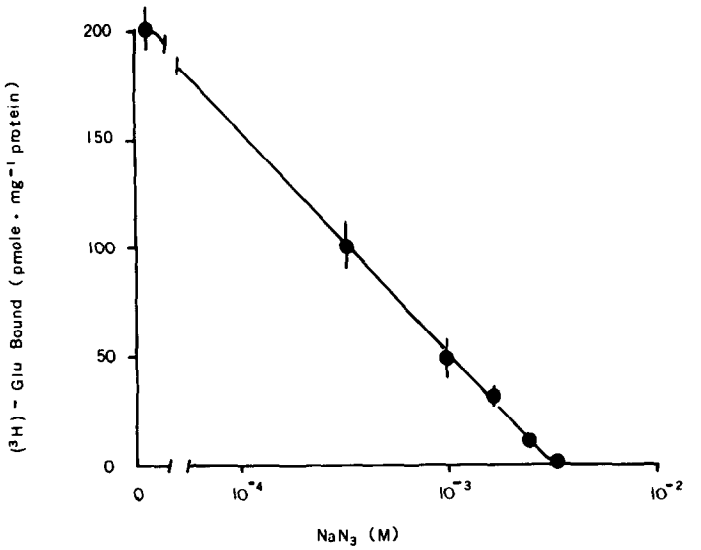

Fig. 1. Effect of NaN_3 on [^{3}H]-L-glutamic acid (80 nM) binding to purified GBP. The protein (4.9 µg) was preincubated with NaN_3 or buffer for 5 min at 23° and the binding assay was initiated by the addition of [^{3}H]-glutamate. Each value is the mean (± S.E.) of four determinations.

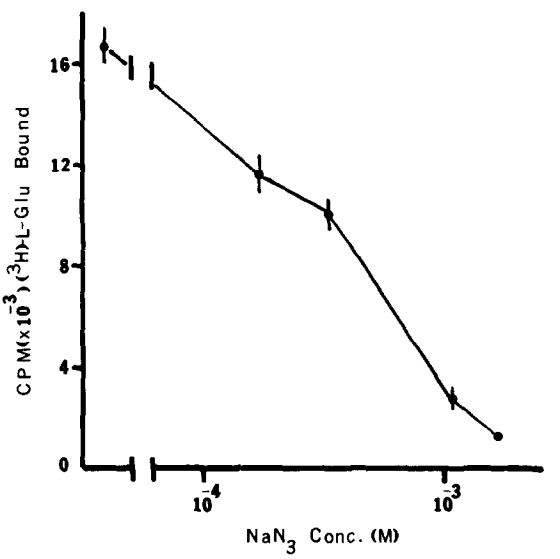

Fig. 2. Effect of NaN_3 on [^{3}H]-L-glutamic acid (80 nM) binding to brain synaptic membranes. All conditions were identical as for Fig. 1. Binding assays were conducted as described in Methods. Each value is the mean (± S.E.) of triplicate determinations.

batches of GBP that were analyzed for iron were also analyzed for their content of labile sulfur, it was determined that they contained 2.19 $\pm$ 0.24 g atom S per mole of GBP. Thus, it would appear that GBP is a metalloprotein which contains an Fe_2S_2 center and that, on the basis of the azide effect on glutamate binding, it is reasonable to suggest that the Fe_2S_2 center is at or near the active site. The procedure used to explore the presence of such a center at the active site of GBP involved the study of the glutamate and azide effects on the difference spectra of GBP. As is shown in Fig. 3A the addition of L-glutamic acid (10^{-6} M) to a preparation of GBP altered the difference spectrum of the protein by increasing its absorbance, with the peak of this change centered around 275 nm. This glutamate-induced increase in absorbance of GBP was dependent on the concentration of the ligand as is shown in Fig. 3B. It is also of interest to note that the addition of NaN_3 following exposure of the protein to 10^{-6} M L-glutamic acid caused a reversal of the glutamate-induced signal with the appearance of a large decrease in absorbance difference at 275 nm (Fig. 3A). Azide by itself at the same concentration (2.7 mM) produced similar decreases in absorbance differences at 275 nm.

It has previously been observed in other studies that metalloproteins with Fe-S centers absorb rather highly at 275 nm frequencies as well as, at some higher frequencies (12,13). It seems possible that the effect of both L-glutamic acid and NaN_3 on the GBP difference spectra are produced on an Fe-S center within the active site of this membrane glycoprotein, although proof of this supposition will require further supportive evidence.

Since the purified binding protein has an almost identical sensitivity to NaN_3 as it does in its native state in the synaptic membranes, it would appear that its Fe-S content is not appreciably altered during purification. As a matter of fact, additions of either $FeSO_4$ or $FeCl_3$ (10^{-6}-10^{-4} M) to the incubation medium of the binding assay had no substantial effect on the binding activity of GBP (Fig. 4 and 5). On the other hand, the addition of similar concentrations of $CuSO_4$ did result in progressive decreases in glutamate binding

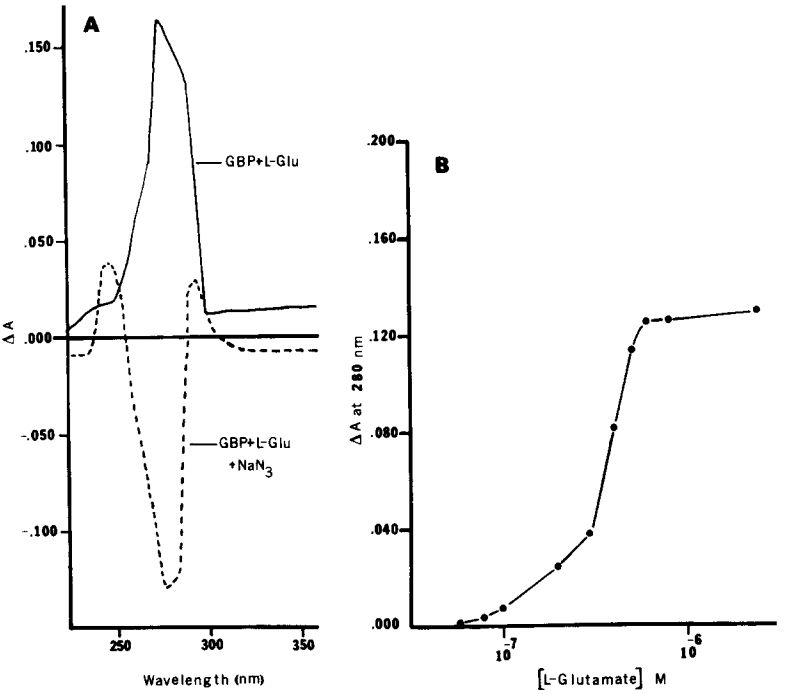

Fig. 3. A) Ultraviolet difference spectrum of GBP (1.0 mg/ml) in the presence of 1.1 μM L-glutamate and in the presence of the same concentration of glutamate plus 2.7 mM NaN_3. The protein solution (1.5 ml in phosphate buffer) was placed in two quartz cuvets and a baseline was obtained from 400 to 200 nm. The spectrum was repeated 5 min after the addition of 1.5 and of 4 μl of the solutions containing L-glutamate and NaN_3, respectively. The reference cell received equal volumes of water.
B) Concentration-dependent increase in glutamate-induced spectral change at 280 nm. Measurements were obtained as described above, 5 min following each glutamate addition.

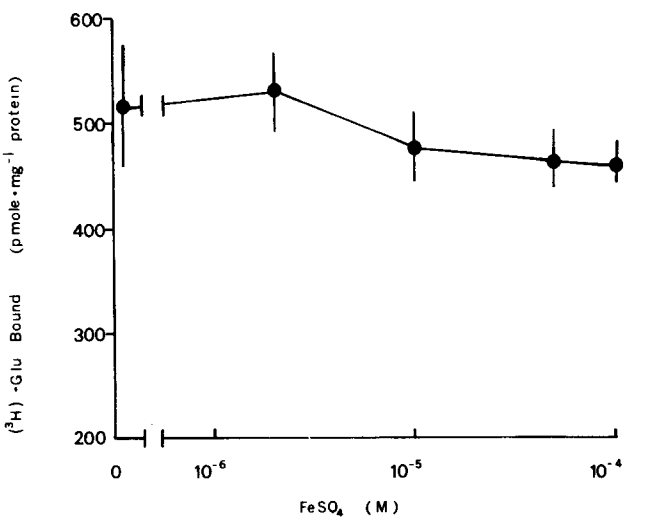

Fig. 4. Effects of increasing concentrations of $FeSO_4$ on [^{3}H]-L-glutamic acid (80 nM) binding to a batch of GBP. The protein (3.1 μg) was preincubated for 5 min with $FeSO_4$ or buffer as described in Fig. 1. Each value is the mean (± S.E.) of four determinations.

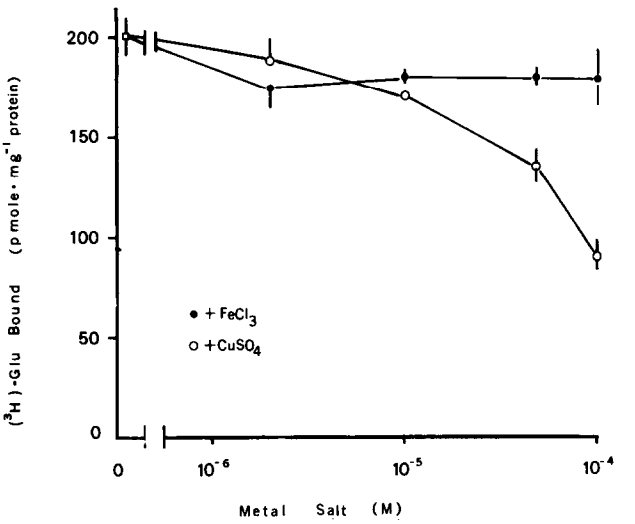

Fig. 5. Effects of $FeCl_3$ and $CuSO_4$ on L-glutamate binding to GBP. The protein (4.9 µg) is from the same batch as that of Fig. 1. Each value is the mean (± S.E.) of four determinations.

(Fig. 5), which would indicate an inhibitory effect by other metals on the activity of this protein.

The results presented in this study are strongly suggestive that the membrane glycoprotein which has many of the characteristics of the physiologic receptor for L-glutamate's neuroexcitatory activity is an iron-sulfur containing protein with an Fe_2S_2 center at the ligand binding site. It remains unclear at this stage whether this Fe_2S_2 center participates in any electron transport reactions at the membrane level besides its apparent function as an excitatory amino acid receptive site.

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REFERENCES

1. Michaelis, E. K., Michaelis, M. L., and Boyarsky, L. L. (1974) Biochim. Biophys. Acta. 367, 338-348.
2. Michaelis, E. K. (1975) Biochem. Biophys. Res. Commun, 65, 1004-1012.

3. Yoshikawa, S. and Orri, Y. (1972) J. Biochem. Tokyo 71, 859-872.
4. Wever, R., Muisjers, A. O., Van Gelder, B. F., Bakker, E. P., and Van Buuren, K. J. H. (1973) Biochim. Biophys. Acta. 325, 1-7.
5. Wever, R. , Muisjers, A. O., and Van Gelder, B. F. (1973) Biochim. Biophys. Acta. 325, 8-15.
6. Curtis, D. R., Johnston, G. A. R., and Duggan, A. W. (1970) Exper. Brain Res. 10, 447.
7. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275.
8. Van De Bogart, M., and Beinert, H. (1967) Anal. Biochem. 20, 325-334.
9. Lowenberg, W., Buchanan, B. B., and Rabinowitz, J. C. (1963) J. Biol. Chem. 238, 3899-3913.
10. Strothkamp, R. E., and Dawson, C. R. (1977) Biochemistry 16, 1926-1929.
11. King, T. E., Ohnishi, T., Winter, D. B., and Wa, J. T. (1976) in Advances in Experimental Biology and Medicine (K. T. Yasunobu, H. F. Mower, O. Hayaishi, Eds.) pp. 182-227, Plenum Press, New York.
12. Matsubara, H., Wada, K., and Masaki, R. (1976) in Advances in Experimental Biology and Medicine (K. T. Yasunobu, H. F. Mower, O. Hayaishi, Eds.) pp. 1-15, Plenum Press, New York.
13. Takemori, S., Suhara, K., and Katagiri, M. (1976) in Advances in Experimental Biology and Medicine (K. T. Yasunobu, H. F. Mower, O. Hayaishi, Eds.) pp. 36-44, Plenum Press, New York.