**Title:** Molecular characteristics of membrane glutamate receptor-ionophore interaction

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**Abstract:** Please see continuation pages.
20. ABSTRACT

The objectives of the research project, supported by the ARO contract shown above, were those of defining the macromolecule or macromolecules that function as the glutamate receptor in synaptic membranes of brain neurons, of demonstrating that functional reconstitution of these sites in a membrane bilayer can be accomplished, and of studying the molecular topography of these sites in such membranes. The studies performed during the tenure of this contract have led to the isolation of a glutamate binding protein from bovine as well as rat brain synaptic membranes, and a complete biochemical characterization of both proteins. Furthermore, antibodies have been raised against these two proteins, and the antibodies against the bovine brain protein have been used in extensive immunochromical and immunocytochemical studies. The immunochromical studies have revealed a high degree of specificity of these antibodies that is associated with a selective inhibition by the antibodies of both glutamate binding to the isolated protein and glutamate-induced ion flux. Substantial progress has been made in the immunochromical labeling of neurons with these antibodies and in tracing the labeled sites at the light and electron microscopic level. Concurrently, a series of studies has been performed to develop the methodology for the reconstitution of a glutamate receptor-like function in liposomes and for the functional reconstitution of a partially or completely purified glutamate binding protein. These studies have led to a successful reconstitution of a glutamate-stimulated ion flux response in such liposomes with a highly purified preparation of the glutamate binding protein. It is our opinion that we have obtained good initial evidence to suggest that the glutamate binding protein that we have isolated is related to some type of receptor activity in brain neurons.

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FOREWORD

An important area of current scientific investigation is the exploration of the nature of the receptors for the excitatory transmitter L-glutamic acid. Based on our current knowledge, L-glutamate is the most wide-spread excitatory system in brain tissue and is thought to be involved in abnormal neurological states such as the appearance of seizures, the neurological damage seen in cerebral ischemia, and possibly the neurological damage associated with such disease states as Huntington's chorea and Alzheimer's dementia. In addition, glutamate receptors may be the target for either the primary or secondary actions of various neurotoxins. It is for these reasons that many investigators are exploring the mechanisms of activation of the glutamate receptors in the central nervous system of mammals and attempting to develop approaches for the inhibition or modulation of the activity of these receptors.

STATEMENT OF THE PROBLEM STUDIED

The area of investigation that we have pursued is related to the molecular characterization of the protein that may be functioning as the receptor site for L-glutamate and for other neuroexcitatory amino acids and toxins. The approaches that we have followed in our studies were:

a) Isolation and characterization of a glutamate binding protein from both bovine and rat brain synaptic membranes.

b) Characterization of the activation of glutamate receptor-like responses, i.e. ion flux activation by glutamate and other excitatory amino acids, in synaptic membranes, in liposome-reconstituted proteins from synaptic membranes, and in liposome-reconstituted preparations of the isolated glutamate binding protein.

c) Development of antibodies against the glutamate binding protein and use of these antibodies to define the distribution and function of this protein, including the elucidation of the molecular topography of the binding protein.

SUMMARY OF THE MOST IMPORTANT RESULTS

a) Isolation and characterization of a brain glutamate binding protein

A glutamate binding protein has been purified in our laboratory from both rat and bovine brain synaptic membranes. Both proteins have been purified to homogeneity as revealed by the fact that a single protein species was obtained on SDS gel electrophoresis, a single protein species was seen on isoelectric focusing, and a single NH₂-terminal amino acid (tyrosine) was detected for both proteins. Co-electrophoresis of these two proteins in SDS gel systems yields a single protein band, i.e. these two proteins are indistinguishable by this procedure. Under conditions for optimal determination of their binding activity, both proteins exhibit similar constants for glutamate binding. These results have been described in two
b) Characterization of glutamate receptor-like responses in synaptic membranes and in liposome-reconstituted protein preparations.

Functionality of glutamate and other excitatory amino acid receptors is defined by their ability to increase membrane permeability for Na⁺ following exposure of these preparations to the amino acids. In our studies we have demonstrated that L-glutamate and other excitatory amino acids, such as D-glutamate, kainate, ibotenate, quisqualate, and NMDA, increase the permeability of synaptic membranes to the flux of Na⁺. This increased influx of Na⁺ was associated with a membrane depolarizing event as determined by the change in distribution of the lipophilic probe [35S]SCN⁻. On the basis of our results with the SCN⁻ probe we have concluded that the receptors activated by the excitatory amino acid NMDA either are different molecular entities or they reside in a different population of membranes than the L-glutamate or kainate activated receptors. This conclusion was based on our observation that whereas L-glutamate and kainate-activated ion flux was not an additive process, the NMDA and L-glutamate-activated ion flux were additive. This would suggest that kainate and glutamate may be sharing either common receptor sites or common ion channel complexes, whereas NMDA receptors may be distinct molecular entities. The results of these studies are described in the publication by Chang, Michaelis and Roy (1984).

In more recent studies we have found that we can solubilize synaptic membranes in the presence of excess lipid (asolecin) and under these conditions we can preserve not only ligand binding activity but also glutamate-induced activation of ion flux. The optimal conditions for the reconstitution of the glutamate activated ion flux response include in addition to the excess lipid presence, the use of non-ionic detergents and the removal of such detergents following solubilization by incubation of the preparations with the polystyrene matrix, Biobeads. Under these conditions of solubilization-reconstitution, 23% of the solubilized synaptic membrane protein is reconstituted into the liposomes and in 80-90% of the experiments we have obtained functional reconstitution of glutamate and kainate-sensitive Na⁺ flux responses. The detergent-solubilized and lipid-protected protein preparations can be processed further for the purification of the glutamate binding protein. We have consistently observed excellent reconstitution of glutamate-activated Na⁺ flux activity following the chromatographic isolation of a fraction highly enriched in glutamate binding activity.

Our initial studies indicated the presence of at least some small quantity of the small molecular weight glutamate binding protein that we had purified previously from rat and bovine brain. However, in most of our most recent studies we have seen enrichment of a protein complex of molecular weight in the range of 60-70 kDa associated with the fractions that exhibited highest glutamate binding activity and glutamate-activated Na⁺ flux (Stormann and Michaelis, unpublished observations). These observations were interpreted in two ways, that either the small molecular weight (M,) glutamate binding protein was not related to the glutamate receptor-ion channel complex or that the small M, protein was a proteolytic degradation fragment of a larger molecular weight protein. This issue will be addressed...
in the succeeding paragraphs.

c) Development of antibodies against the glutamate binding protein and use of the antibodies as molecular probes.

The glutamate binding protein purified from bovine brain was used to develop antisera against this protein. The specificity of the antisera was examined by the enzyme-linked immunosorbent assay procedure (ELISA). The antibody binding protein antisera were found to be highly selective for this glutamate binding protein, showing essentially no cross-reactivity against any of the other glutamate-metabolizing enzymes, except for a low level of cross-reactivity against glutamate decarboxylase. These observations have been described in publications by Roy and Michaelis, 1984, Roy et al., 1985, Michaelis et al., 1986, and in the dissertation by S. Roy.

The anti-glutamate binding protein antibodies did not have any effect on either glutamate transport carriers in synaptic membranes or depolarization-activated glutamate releasing sites in synaptic membranes. However, the antibodies did inhibit glutamate binding to the purified protein and glutamate activation of Na⁺ flux. These antibodies also inhibited kainic acid induced ion flux as well as quisqualate-induced flux, albeit that the inhibition of these two putative receptor-ion channel complexes was 10-60 times less sensitive than the inhibition of ion flux produced by L-glutamate. The antibodies had no effect on the ion flux brought about by the other major excitatory amino acid, NMDA. Based on these observations we have concluded, once again, that the L-glutamate, kainate and quisqualate receptor complexes may be related macromolecular entities, whereas the NMDA receptors detected in our assays are probably different macromolecular species.

In our most recent studies using immunoblotting procedures, we have found that the antibodies that we have raised react very strongly with a synaptic membrane protein of estimated molecular weight equal to 70 kDa on SDS gel electrophoresis. This protein is rather readily degraded by proteolytic enzymes to the 15 kDa protein that we had previously identified as the glutamate binding protein. We have recently purified the 70 kDa protein in the presence of five protease inhibitors and have shown that the completely pure, homogeneous protein is a 68-70 kDa glutamate binding protein. The ligand binding characteristics of this protein are identical to those we had previously described for the smaller Mr glutamate binding protein. In addition, when this 70 kDa protein is reconstituted into liposomes exhibits excellent responses to glutamate in initiating Na⁺ flux. Finally, this protein has no glutamate metabolizing activity. Our current investigations are focused on demonstrating the relationship of this 70 kDa protein to the intact glutamate receptor complex in synaptic membranes and to the previously identified small Mr glutamate binding protein.

CONCLUSIONS

We believe that our studies may yield the first complete purification of an excitatory amino acid receptor protein that may be used to model studies...
on the activity of these very important brain transmitters. In addition, we feel that we are at the threshold of developing the techniques for using this glutamate binding protein in screening or detection systems for the evaluation of the toxicity of neurochemical toxins and other lethal or incapacitating agents.

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Dr. Elias K. Michaelis, Professor of Human Development and Biochemistry
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Articles Published


