Antibodies to a C-terminal peptide of the rat brain glutamate receptor subunit, GluR-A, recognize a subpopulation of AMPA binding sites but not kainate sites

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Antibodies were made to a thirteen amino acid synthetic peptide corresponding to the C-terminal portion of the glutamate (glu) receptor, GluR-A. The immunoprecipitation of kainic acid (KA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) binding sites by the anti-peptide antibodies was studied using a detergent-solubilized preparation of rat brain membranes. Under these conditions a subpopulation of AMPA binding sites was recognized by the antibodies, but no KA binding sites were recognized. Scatchard analysis of this subpopulation of AMPA binding sites yields a curvilinear plot which fits a two-site model with dissociation constants of 4.6 and 323 nM. These studies show that the glu receptor complex, GluR-A, binds AMPA but not KA and suggest that (i) the binding sites for these two ligands reside on different proteins, and (ii) the KA receptor identified physiologically is not equivalent to the KA binding sites identified with ^{3}H-labelled KA.

Excitatory amino acid receptor; Receptor immunoprecipitation; Glutamate; Neurotransmitter

1. INTRODUCTION

Excitatory amino acids (EAA) are major neurotransmitters in the mammalian brain and have been implicated in several critical neurological events including neurotoxicity, neurodegenerative disorders and learning and memory [1]. EAA receptors have been characterized with respect to their physiological responses to agonists and antagonists and their binding properties using radiolabeled ligands. Both approaches have identified three major populations of receptors: the *N*-methyl-D-aspartate (NMDA), quisqualate or α-amino-3-hydroxy-5-methyl-4 isoxazolepropionic acid (AMPA), and kainic acid (KA) receptors [1,2]. While both binding and physiological studies clearly differentiate between NMDA and non-NMDA receptors, KA and AMPA receptors are not well defined. Several studies have suggested that the physiologically active sites for these two agonists may be the same or associated with the same receptor complex [3-7]. Both KA and AMPA bind to mammalian brain with high affinity [2,8-11] and their binding sites have different distributions [12,13]. However, the relationship between the high affinity binding sites for KA and AMPA and the physiologically active receptors of these agonists has not been conclusively demonstrated. A glutamate (glu) receptor from rat brain has been cloned through functional expression using the *Xenopus* oocyte for expression of mRNA [14]. The physiological properties of the oocyte-expressed receptor suggest that it is a KA subtype of excitatory amino acid receptor. However, this receptor (GluR-A) and 3 related receptors have recently been shown to bind AMPA when expressed in cultured mammalian cells [15]. To further characterize this receptor, we have studied the binding properties of GluR-A in a detergent-solubilized preparation from rat membranes using antibodies to a C-terminal peptide of the protein.

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2. MATERIALS AND METHODS

KA and AMPA binding sites were solubilized from rat brain membranes with Triton X-100 as previously described [8], except that the temperature of the solubilization step was 4°C instead of 37°C. Under these conditions both solubilized KA and AMPA binding sites were obtained. The solubilized preparation was dialyzed extensively in THCl buffer (50 mM Tris-HCl, pH 7.0,10% (w/v) glycerol and 0.1% (w/v) Triton X-100). All subsequent experiments with the solubilized binding sites were done in this buffer. [^{3}H]AMPA binding was routinely done at 10 nM in the presence of 0.1 M KSCN as described [8]. For Scatchard analysis of [^{3}H]AMPA binding, concentrations ranging from 5 to 500 nM were used. Data were analyzed using the Ligand program, a computerized non-linear curve-fitting program [16]. In competition experiments the concentration of competing compound inhibiting 50% of the binding activity (IC_{50}) was determined by logit-log analysis [17]. [^{3}H]KA binding was done at 5 and 50 nM as described [18].

A synthetic peptide, SHSSGMPLGATGL (single letter code), which corresponds to the C-terminal of GluR-A (initially called GluR-K1 [14]) was prepared commercially. Of the four glu receptors whose sequence has been determined, this sequence is specific to GluR-A [15,19]. However, this sequence overlaps to a limited extent with the C-terminal sequence of GluR-D, RQSSGLAVIASDLP. Therefore,

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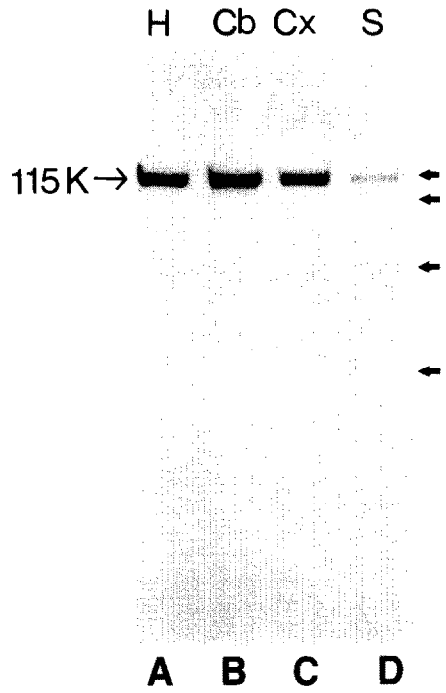

Fig. 1. Immunoblot analyses of SDS gels of rat brain tissues using anti-peptide antibodies. A, hippocampus; B, cerebellum; C, cortex; D, brain-stem. Twenty-five micrograms of protein was applied to each lane. Arrows show the positions of molecular weight standards β-galactosidase, $M_r = 116000$; phosphorylase b, $M_r = 97000$; bovine serum albumin, $M_r = 67000$; and ovalbumin, $M_r = 44000$.

we cannot rule out the possibility that GluR-D is recognized by antibodies to the C-terminal peptide of GluR-A. The peptide was conjugated to BSA with glutaraldehyde, and after dialysis, the conjugate was injected into New Zealand White rabbits for producing antibodies. Antibodies were attached to Protein A-agarose, and after thorough washing to remove serum components, columns of the immobilized antibody (100 μl bed volume) were used to determine antibody recognition of the solubilized AMPA and KA binding sites [20]. Aliquots of the solubilized preparation were applied to the columns and incubated at 4°C for 1.5 h. [^{3}H]AMPA and [^{3}H]KA binding were measured in the unbound fractions from columns containing anti-peptide antibodies and compared to those prepared with preimmune serum or normal rabbit serum. [^{3}H]AMPA binding and [^{3}H]KA binding were done on the same fractions. For immunopurification of AMPA binding sites, antibodies were covalently attached to Protein A-agarose [21]. To a column containing 0.6 ml of resin, 10 ml of solubilized preparation was applied and incubated for 1.5 h at 4°C. After extensive washing with buffer, bound antigen was eluted with 10 ml of 1 M KSCN. [^{3}H]AMPA binding was carried out on this fraction after dialysis.

SDS-PAGE was performed according to the method of Laemmli [22] using gradient gels of 4–20% polyacrylamide. After electrophoresis, transfer to nitrocellulose membranes was done as described by Towbin [23]. Membranes were treated with 4.5% instant powdered milk in Tris-buffered saline (TBS; 50 mM Tris, 150 mM NaCl, pH 7.4) containing 0.05% Tween-20, followed by incubation in antibody at a dilution of 1/1000 in the same buffer. Detection was carried out using an alkaline phosphatase-conjugated second antibody.

3. RESULTS

Immunoblot analysis of SDS gels of rat brain using the anti-peptide antibody shows a single band migrating with a $M_r = 115000$ in the four brain areas analyzed (Fig. 1). The size of the immunoreactive protein is consistent with the data from the cDNA clone for GluR-A which encodes a protein with a calculated molecular mass of 99800 Da [15]. The slightly larger molecular mass we obtain may be due to glycosylation of the protein since GluR-A contains 6 possible glycosylation sites [14]. The relative amounts of immunoreactive protein is also consistent with in situ hybridization studies and Northern blot analyses showing high concentrations of GluR-A mRNA in cortex, cerebellum and hippocampus, and relatively little in brain stem [14,15].

Immunoprecipitation studies were carried out on rat brain membranes solubilized in Triton X-100 under conditions which give soluble binding sites for both AMPA and KA [8,18]. This allows the binding properties of both ligands to be directly compared on the same fractions. Solubilized preparations were passed through columns of anti-peptide antibody coupled to Protein A-agarose [20] and antibody binding was assessed by determining the reduction in [^{3}H]AMPA or [^{3}H]KA binding in the unretained fraction, relative to controls. Using this protocol we find that a subpopulation (27–30%) of AMPA binding sites is removed by the antibodies, while no KA binding activity is removed (Table I). Most studies have demonstrated two KA binding sites in mammalian brain, a high affinity site with a dissociation constant between 1–8 nM and a low affinity site with a dissociation constant between 27–65 nM [2]. Since it has been suggested that the high and low affinity sites may be on different proteins [3], ^{3}H-KA binding was done at both 5 and 50 nM. Increasing the amount of antibody attached to the column did not increase the amount of AMPA binding activity removed, confirming that only a subpopulation of AMPA binding sites is recognized by the antibody. Varying the amount of Protein A-agarose also did not change the amount of AMPA binding removed (unpublished observation). These findings show that the binding sites

Table I

Immunoprecipitation of AMPA and KA binding sites with anti-peptide antibodies

Volume of serum	Per cent Binding Recovered		
	AMPA	KA	
		5 nM	50 nM
100 μl	72.4 ± 4.8	96.8 ± 6.3	98.8 ± 4.2
250 μl	70.7 ± 1.6	98.7 ± 3.9	nd

Data are presented as per cent of binding activity unretained by a column of Protein A-agarose to which anti-peptide antibody, 100 μl or 250 μl serum, was bound relative to that of a column containing preimmune serum or normal rabbit serum (mean ± SD; $n = 5$ for 100 μl and $n = 3$ for 250 μl). AMPA and KA binding were measured in the unbound fraction from the same column. [^{3}H]AMPA binding was done at 10 nM in the presence of 0.1 M KSCN. nd, not determined.

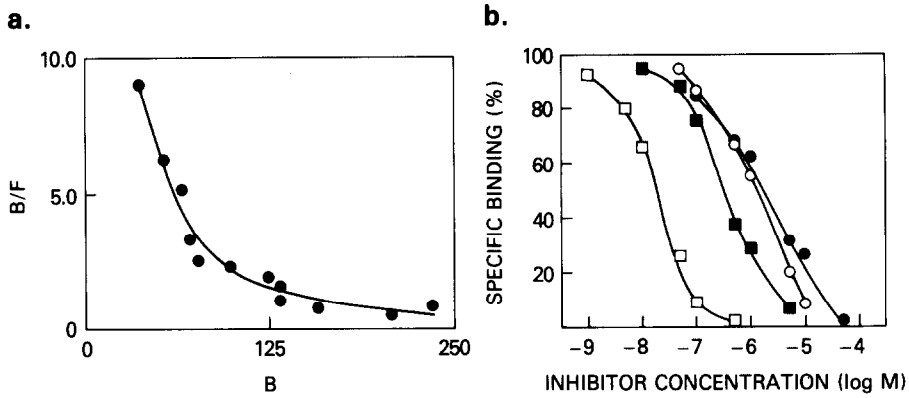

Fig. 2. (a) Scatchard analysis of [^{3}H]AMPA binding to the fraction retained on Protein A-agarose to which anti-peptide antibody was bound. Data were analyzed using the LIGAND program [16] and found to fit a two-site model. Dissociation constants of 4.6 ± 1.7 nM and 323 ± 186 nM were obtained for the high and low affinity sites, respectively (mean ± SEM of 3 determinations). Bound are expressed as fmol/assay and bound/free are expressed as fmol/assay/nM. (b) Inhibition profiles of quisqualate, glutamate, 6,7-dinitroquinoxaline-2,3-dione (DNQX) and KA for [^{3}H]AMPA binding to fraction retained on Protein A-agarose. [^{3}H]AMPA binding was done at 10 nM. IC_{50} values (mean ± SD of 3 determinations) obtained for quisqualate, glutamate, DNQX and KA were 0.018 ± 0.004, 0.40 ± 0.09, 0.99 ± 0.16 and 2.0 ± 0.4 μM, respectively. Figure a and b each show the average of 3 determinations.

for KA (both high and low affinity sites) and AMPA in rat brain are associated with different proteins.

To characterize the subpopulation of AMPA binding sites recognized by the antibody, the anti-peptide antibody was covalently attached to the Protein A-agarose [21]. Antigen bound to the column was eluted with 1 M KSCN, and [^{3}H]AMPA binding was carried out on this fraction after dialysis. A curvilinear Scatchard plot is obtained which fits a two-site model with dissociation constants of 4.6 and 323 nM (Fig. 2). With respect to the presence of two binding sites, these results are similar to those found measuring the total population of AMPA binding sites that are either membrane associated [8,9-11] or detergent solubilized [8]. However, the values of the dissociation constants are somewhat lower than those obtained for the total solubilized rat brain preparation (11.9 and 597 nM) [8]. Quisqualate, L-glutamate, 6,7-dinitroquinoxaline-2,3-dione (DNQX) and KA inhibited AMPA binding to the antibody-purified preparation (Fig. 2) at concentrations similar to those that inhibit binding to the total population of AMPA binding sites. Specific KA binding was also measured in the antibody-purified preparation and could not be detected at concentrations up to 1.5 μM (unpublished observation) suggesting that a lower affinity binding site, such as that found in chick brain (K_d = 255 nM) [24], is also not present.

4. DISCUSSION

Our finding that antibodies to the C-terminal of the glu receptor subunit, GluR-A, recognize AMPA binding sites, but not KA binding sites, has three interpretations. (i) GluR-A is an AMPA binding protein which does not bind KA. (ii) GluR-A is subunit of a receptor complex which also contains an AMPA binding subunit and the entire complex is adsorbed by antibodies to GluR-A. If this is the case, the lack of KA binding to the complex remains unexplained. (iii) A significant portion of the C-terminal amino acid sequence of GluR-A used to produce the antibody is found both in GluR-A and in a distinct AMPA binding protein, but the antibody cannot bind GluR-A due to modifications of the C-terminal portion of the receptor. We consider the most likely interpretation to be that the KA receptor, GluR-A, is an AMPA binding protein which does not bind KA under the standard conditions used to measure its binding to mammalian brain. This is supported by the recent finding that, when expressed in mammalian cultured cells, GluR-A and related receptors expressed AMPA binding sites and not KA binding sites [15]. Results using the transfected cells showed only a single class of binding sites with a dissociation constant of 12 nM which is similar to that of the high affinity site (4.6 nM) we observed for the antibody-purified receptor. However, a low affinity site, which we found for the antibody-purified receptor and which has been reported in many AMPA binding studies using mammalian brain membranes, is not present in the transfected cells. There are a number of explanations for this difference including the possibility that GluR-A undergoes different posttranslational processing in neurons and cultured cells.

The present results imply that the KA receptor and KA binding sites are distinct proteins in mammalian brain, as has been suggested by other studies. The strongest previous evidence supporting this contention is that rat brain KA receptors expressed in *Xenopus* oocytes show potencies of agonists for KA receptors that are 1000-fold lower than those predicted from binding data, and the relative order of potencies of agonists applied to oocytes differs from that of binding

studies [25]. If KA receptors and KA binding sites are distinct, the question as to the nature and function of KA binding sites in mammalian brain remains. These sites are of low abundance compared to AMPA binding sites and may represent a minor population of excitatory amino acid receptors. Recently cDNA clones for KA binding proteins from chick [26] and frog [27] brain have been isolated. These proteins bind KA, with the frog protein being pharmacologically indistinguishable from that of mammalian brain, but they have not been shown to be functional ion channels. However, the KBP's have several characteristics of a ligand-gated ion channel and have significant homology (35% amino acid identity) to the C-terminal half of GluR-A. A similar protein may be responsible for KA binding in mammalian brain, and the function of this protein remains to be determined.

REFERENCES

[1] Monaghan, D.T., Bridge, R.J. and Cotman, C.W. (1989) Annu. Rev. Pharmac. Tox. 29, 365-402.
[2] Foster, A.C. and Fagg, G.E. (1984) Brain Res. Rev. 7, 103-164.
[3] Honore, T., Drejer, J. and Nielsen, M. (1986) Neurosci. Lett. 65, 47-52.
[4] Ishida, A.T. and Neyton, J. (1985) Proc. Natl. Acad. Sci. USA 82, 1837-1841.
[5] O'Brien, R.J. and Fishbach, G.D. (1986) J. Neurosci. 6, 3275-3283.
[6] Rassendren, F.A., Lory, P., Pin, J.P., Bockaert, J. and Nargeot, J. (1989) Neurosci. Lett. 99, 333-339.
[7] Pin, J.P., Van Vliet, B.J. and Bockaert, J. (1989) Eur. J. Pharmacol. 172, 81-91.
[8] Hunter, C., Wheaton, K.D. and Wenthold, R.J. (1990) J. Neurochem. 54, 118-125.
[9] Honore, T. Lauridsen, J. and Krogsgaard-Larsen, P. (1982) J. Neurochem. 38, 173-178.
[10] Murphy, D.E., Snowhill, E.W. and Williams, M. (1987) Neurochem. Res. 12, 775-782.
[11] Olsen, R.W., Szamraj, O. and Houser, C.R. (1987) Brain Res. 402, 243-254.
[12] Monaghan, D.T., Holets, V.R., Toy, D.W. and Cotman, C.W. (1983) Nature 306, 176-179.
[13] Monaghan, D.T., Yao, D. and Cotman, C.W. (1985) Brain Res. 324, 160-164.
[14] Hollman, M. O'Shea-Greenfield, A., Rogers, S.W. and Heinemann, S. (1989) Nature 342, 643-648.
[15] Keinanen, K., Wisden, W., Sommer, B., Werner, P., Herb, A., Verdoorn, T.A., Sakamnn, B. and Seeburg, P.H. (1990) Science 249, 556.
[16] Munson, P.J. and Rodbard, D. (1980) Anal. Biochem. 107, 220-239.
[17] Rodbard, D. and Frazier, G.R. (1975) Methods Enzymol. 37, 3-22.
[18] Hampson, D.R., Huie, D. and Wenthold, R.J. (1987) J. Neurochem. 49, 1209-1215.
[19] Boulter, J., Hollmann, M., O'Shea-Greenfield, A., Hartley, M., Deneris, E., Maron, C. and Heinemann, S. (1990) Science 249, 1033-1037.
[20] Hampson, D.R., Wheaton, K.D., Dechesne, C.J. and Wenthold, R.J. (1989) J. Biol. Chem. 264, 13329-13335.
[21] Schneider, C., Newman, R.A., Sutherland, D.R., Asser, U. and Greaves, M.F. (1982) J. Biol. Chem. 257, 10766-10769.
[22] Laemmli, U.K. (1970) Nature 227, 680-685.
[23] Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350-4354.
[24] Gregor, P., Eshhar, N., Ortega, A. and Teichberg, V.I. (1988) EMBO J. 7, 2673-2679.
[25] Verdoorn, T.A. and Dingledine, R. (1988) Mol. Pharmacol. 34, 298-307.
[26] Gregor, P., Mano, I., Maoz, I., McKeown, M. and Teichberg, V.I. (1989) Nature 342, 689-692.
[27] Wada, K., Dechesne, C.J., Shimasaki, S., King, R.G., Kusano, K., Buonanno, A., Hampson, D.R., Banner, C., Wenthold, R.J. and Nakatani, Y. (1989) Nature 342, 684-689.