0026-895X/92/020203-07$03.00/0

MOLECULAR PHARMACOLOGY, 42:203-209

Biochemical Characterization of Kainate Receptors from Goldfish Brain

CYNTHIA J. ZIEGRA, JAMES M. WILLARD, and ROBERT E. OSWALD
Department of Pharmacology, College of Veterinary Medicine, Cornell University, Ithaca, New York 14853
Received March 12, 1992; Accepted May 14, 1992

SUMMARY

Kainate receptors from goldfish brain were purified by affinity chromatography. Unlike previously published purifications, which have yielded single proteins of 48–50 kDa from frog, chick, and pigeon brain, our preparations contained two polypeptides, of 41 kDa and 45 kDa. In addition, a broad band centered at 120 kDa was present. Some of the 41-kDa and 45-kDa polypeptides were derived from the higher molecular mass protein. All of these proteins were recognized by a monoclonal antibody produced against a purified frog kainate receptor. The distribution of the 41-kDa and 45-kDa proteins varied independently in different major brain regions, suggesting that they can exist as separate independent proteins. A partial amino acid sequence of the 41-kDa polypeptide is very similar (40–60% identity) to specific segments of the frog and chick kainate-binding proteins and the α-amino-3-hydroxy-5-methylisoxazolepropionate/kainate ion channels. The characteristics of the 41-kDa and 45-kDa polypeptides suggest that these two proteins are distinct. Photoaffinity labeling with [^{3}H]kainate showed that a [^{3}H]kainate binding site is associated with the 41-kDa polypeptide, and peptide mapping suggests that the two proteins are not identical. In addition, the two peptides do not appear to be related by differential glycosylation or phosphorylation. The 41-kDa and 45-kDa polypeptides, therefore, appear to be distinct and may represent kainate receptor subtypes or, in some cases, possibly two different subunits of a kainate receptor complex.

In the CNS, L-glutamate and related amino acid analogs function as the predominant mediators of excitatory neurotransmission (1). Originally, glutamate-activated ion channels were identified and classified according to the agonists (kainate, quisqualate, and NMDA) by which these cation channels are activated (2). The cDNAs for several glutamate receptors have been cloned and sequenced. These include the following. First is a series of cDNAs coding for proteins, from mammalian brains, with molecular weights of approximately 100,000 (3–6). Most of these are ligand-gated ion channels, activated by micromolar concentrations of kainate and AMPA; however, AMPA binds to these receptors with much higher affinity than does kainate (5). Recently, two additional related proteins have been described. One is an ion channel activated by kainate but not AMPA (7), and the other is a high affinity kainate receptor with no presently known function (8). A high degree of homology is present among these proteins (40–80% identity). Second are cDNAs, from frog and chick brain, coding for proteins with molecular weights of approximately 50,000 (9, 10), which bind kainate with high affinity. The function of these low molecular weight glutamate receptors is presently unknown. These two kainate-binding proteins are highly homologous (55% identity) and have approximately 35% identity with the carboxyl-terminal portion of the glutamate receptors described above. Third is a family of cDNAs, cloned from rat brain, coding for second messenger-linked quisqualate receptors (11). The second messenger-linked quisqualate receptors have low (16% identity) but significant homology with the other mammalian glutamate receptors. These results indicate that glutamate receptors, like acetylcholine and γ-aminobutyric acid receptors, exist as subtypes that either serve as integral ligand-gated ion channels or act through G protein-coupled second messenger pathways.

Kainate is a cyclic analog of L-glutamate and is a potent neurotoxin that was originally isolated from the marine algae *Digenea simplex*. It evokes a wide variety of actions in the CNS, the most well characterized of which is the gating of Na^+ and K^+ ion conductances (1). These kainate-activated ionotropic receptors mediate excitatory postsynaptic potentials throughout the CNS and are also thought to be involved in the neurotoxic effect of kainate (12). In addition, kainate has also been shown to evoke cGMP (13) and nitric oxide production (14),

This work was supported by Grants BNS-851956 and BNS-9010229 from the National Science Foundation, to R.E.O. C.J.Z. was supported by a fellowship from the Cornell Biotechnology Institute.

ABBREVIATIONS: CNS, central nervous system; AMPA, α-amino-3-hydroxy-5-methylisoxazolepropionate; CNQX, 6-cyano-7-nitroquinoxoline-2,6-dione; DTT, dithiothreitol; EDAC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; G protein, heterotrimeric guanine nucleotide-binding protein; Gpp(NH)p, guanosine 5′-(β,γ-imino)triphosphate; GTPγS, guanosine 5′-*O*-(3-thio)triphosphate; HMEN buffer, 20 mM *N*-(2-hydroxyethyl)-piperizine-*N*′-2-ethanesulfonic acid, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA; NMDA, *N*-methyl-D-aspartate; octylglucoside, *n*-octyl-β-D-glucopyranoside; PIPES, piperazine-*N*,*N*′-bis[2-ethanesulfonic acid]; SDS, sodium dodecyl sulfate.

the activation of Ca^{2+} influx (probably through voltage-sensitive Ca^{2+} channels) in both astrocytes (15, 16) and neurons (17), and the reduction of certain hippocampal K^+ conductances (18). In mammals, at least a portion of the monovalent cationic channel activity is mediated through a series of related 100-kDa AMPA/kainate receptors (3–5). Homologous proteins, showing ion channel activity, have not yet been described in nonmammalian vertebrates, although Ishida and Neyton (19) have shown that kainate activates cation conductances in goldfish retina.

The specific signal transduction pathways mediating kainate-evoked physiological responses other than the direct opening of cation channels have not yet been fully described. Previously, we have shown that at least some of the kainate-binding proteins in goldfish synaptic plasma membranes interact with pertussis toxin-sensitive G proteins (20). Additionally, we have purified a kainate receptor (21) by agonist affinity chromatography (22) and found that the receptor copurifies with a G_o-like protein. In these preparations, [^{3}H]kainate binding affinity is decreased in the presence of guanine nucleotides, presumably due to a dissociation of the kainate receptor-G protein complex. Also, kainate stimulates [^{3}H]Gpp(NH)p binding and [γ-^{32}P]GTP hydrolysis. When the receptor, separated from the endogenous G protein, is reinserted into phospholipid vesicles with purified bovine G_o, kainate can stimulate [γ-^{32}P] GTP hydrolysis. These data suggest that some of the biochemical responses to kainate may be mediated through a G protein-linked second messenger pathway (reviewed in Ref. 23).

Kainate-binding proteins from frog, chick, and pigeon have been purified (22, 24, 25), and SDS-polyacrylamide gels of these purified preparations show single polypeptides of 48–50 kDa. We have been able to separate the goldfish kainate receptor from its endogenous G protein during purification by incubating and washing the adsorbed soluble brain extracts with GTPγS before elution (21). This purification scheme yields a 41/45-kDa doublet on silver-stained SDS-polyacrylamide gels. On Western blots, both proteins are recognized by a monoclonal antibody raised against the purified frog kainate-binding protein (KAR-B1) (see Ref. 26). In the present study, we have further characterized these two proteins and present evidence that, although both share a common epitope with the frog receptor, these two bands represent distinct proteins.

Experimental Procedures

Materials. Goldfish (*Carassius auratus*) were purchased from Grassyfork Fisheries (Martinsville, IN). [^{3}H]Kainate (58 Ci/mmol) was obtained from New England Nuclear (Wilmington, DE). Octylglucoside was from Calbiochem Co. (La Jolla, CA). AH-Sepharose 4B was from Pharmacia LKB Biotechnology, Inc. EDAC and electrophoresis reagents were obtained from Bio-Rad (Richmond, CA). GTPγS, endoglycosidase F/*N*-glycosidase F, endoproteinase Glu-C (sequencing grade, *Staphylococcus aureus* V8 protease), and chymotrypsin were purchased from Boehringer Mannheim Biologicals (Indianapolis, IN). The monoclonal antibody KAR-B1 was a gift from Dr. Robert J. Wenthold (National Institutes of Health). The potato acid phosphatase was donated by Dr. David Shalloway (Cornell University). CNQX was a gift from Dr. Linda Nowak (Cornell University). The remaining chemicals, including kainate and domoate, were from Sigma Chemical Co. (St. Louis, MO).

Purification of kainate receptor. In all steps, the buffers contained the protease inhibitors 0.2 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 1 μg/ml leupeptin. Goldfish synaptosomal membranes, prepared as described for whole brain (20), were solubilized with 2% octylglucoside (final detergent/protein ratio of 20:1) in HMEN buffer. This mixture was incubated for 1 hr and then centrifuged for 1 hr at 100,000 × *g*. The resulting supernatant was applied, at a rate of 0.15 ml/min, to a 5-ml domoic acid affinity column (equilibrated in solubilization buffer), prepared as described by Hampson and Wenthold (22). The column was washed with 200 ml of HMEN containing 1.5% octylglucoside (wash buffer), at a rate of 0.15 ml/min. The column was brought to room temperature and, except where indicated, 1 column volume of 100 μM GTPγS in HMEN/octylglucoside was applied, followed, after a 20-min incubation, by 20 ml of 100 μM GTPγS, at 0.15 ml/min. This step was done in order to separate the associated G proteins from kainate receptors (21). The column was equilibrated with wash buffer (20 ml). After a 10-min equilibration with 1 column volume of 10 mM kainate in HMEN/octylglucoside, the column was eluted with 20 ml of this buffer, at a rate of 1 ml/min. The eluate was collected on ice and concentrated 10–20-fold with Centricon microconcentrators (Amicon, Beverly, MA) before further use.

[^{3}H]Kainate binding assays. Purified eluates were dialyzed against 500 ml of HMEN buffer containing 1.5% cholate (six changes over a period of approximately 48 hr), to remove kainate. Reconstitution into phospholipid vesicles was as previously described (27). Briefly, 100 μl of sonicated asolectin vesicles (17 mg/ml) were added to 400 μl of purified/dialyzed receptor [lipid to protein ratio (w/w) of 50–100:1] and incubated on ice for 30 min. Detergent was removed by Extractigel D chromatography, and aliquots were used immediately or stored in liquid nitrogen for future use. [^{3}H]Kainate binding to purified reconstituted receptor was measured as described previously for membranes (20). The additional presence of 100 μM kainate in the assays defined nonspecific binding.

Photoaffinity labeling. In a total volume of 100 μl, purified and reconstituted aliquots of receptor (3–5 μg of protein) were incubated at room temperature with 350 nM [^{3}H]kainate, in HMEN buffer, for 15 min. The reaction mixtures were subjected to UV irradiation (254 nm; Mineralight, Ultraviolet Products, Inc.) for 3 min, at a distance of 4 cm. The reaction was terminated by the addition of 25 μl of 5× SDS sample buffer (final concentration of 0.0625 M Tris-HCl, 2% w/v SDS, 10% v/v glycerol, pH 6.8). These samples were warmed to 60° for 5 min and separated by electrophoresis in 8 or 10% SDS-polyacrylamide gels. The gels were stained with Coomassie blue, soaked for 15 min in Autofluor (National Diagnostics), and dried. Kodak XAR-5 film, preflashed to an absorbance of 0.2, was exposed to gels, in cassettes with intensifying screens, for 3–6 weeks at −80°.

Gel electrophoresis and immunoblotting. For SDS-polyacrylamide gel electrophoresis, 40-μl samples were mixed with 10 μl of 5× SDS sample buffer and were heated to 60° for 5 min, and proteins were separated on 8, 10, or 12% minigels. β-Mercaptoethanol (8%) was included in all samples, except when indicated. For silver staining, gels were fixed with 5% glutaraldehyde. For immunoblotting, proteins were electrophoretically transferred to Immobilon membranes (Millipore, Bedford MA). The membranes were then incubated for 1 hr, at room temperature, in blocking buffer [50 mM Tris-citrate, pH 7.2, containing 0.1% Tween-20 and 4.5% (w/v) instant powdered milk]. The membranes were incubated overnight with a 1/25 dilution (in the same buffer) of the culture supernatant containing KAR-B1 (26). After two (10-min) washes in 50 mM Tris-citrate, pH 7.2, containing 0.1% Tween-20, and one wash in blocking buffer, these membranes were incubated for 2 hr with an alkaline phosphatase-conjugated goat anti-mouse secondary antibody, diluted 1/5000 in blocking buffer. Polypeptides recognized by KAR-B1 were visualized using alkaline phosphatase substrates.

Peptide mapping. Purified proteins (5–10 μg of protein) were separated on 8% SDS-polyacrylamide gels (1.5 mm) and were briefly stained with 0.1% (w/v) Coomassie blue (5-min stain and destain). The 41- and 45-kDa protein bands were excised and partially proteolyzed, by the method of Cleveland *et al.* (28), on 12% SDS-polyacrylamide

gels. Either endoproteinase Glu-C (sequencing grade *S. aureus* V8 protease; 3.8 μg/lane) or chymotrypsin (7.5 μg/lane) was used for proteolysis. Peptides were visualized by silver staining and by immunoblotting.

Deglycosylation and dephosphorylation. To deglycosylate, purified and reconstituted proteins were boiled for 2 min in 1% SDS and then diluted 10-fold with buffer consisting of 20 mM sodium phosphate (pH 6.0), 10 mM EDTA, and 0.5% octylglucoside. The samples were boiled for an additional 2 min and allowed to cool. Aliquots (0.05–0.1 μg of protein) were incubated for 16 hr at 37°, in the presence and absence of 0.5 units of endoglycosidase F/*N*-glycosidase F. To dephosphorylate, purified reconstituted proteins (0.05–0.5 μg of protein) were incubated at 37° for 1 hr, in 40 mM PIPES (pH 6.0), 1 mM DTT, in the presence and absence of 4 μg of potato acid phosphatase. Proteins were separated on 10% SDS-polyacrylamide gels and were visualized by silver staining and immunoblotting.

Amino acid sequencing. Affinity-purified proteins (50 μg of protein) were separated on 8% SDS-polyacrylamide gels. The bands were individually excised and digested with 3.75 μg of *S. aureus* V8 protease on a second gel, by the method of Cleveland *et al.* (28). Peptides were electrophoretically transferred to Immobilon membranes (Millipore). Bands were visualized with Coomassie blue stain and were excised and microsequenced by the Cornell Biotechnology Sequencing Facility.

Protein analyses. The protein content of samples or bovine serum albumin standards was determined using a bicinchoninic acid protein assay kit from Pierce (Rockford, IL).

Results

The kainate receptor can be purified from goldfish whole-brain synaptic membranes by using domoic acid affinity chromatography (21). SDS-polyacrylamide gel electophoresis of purified eluates consistently revealed two protein bands, of 45 and 41 kDa (Fig. 1, *lane A*). The 41-kDa band was more intensely stained with silver and Coomassie blue. In addition, a broad band centered at approximately 120 kDa, often with more well defined bands at 115 kDa and 135 kDa, was generally observed. The monoclonal antibody KAR-B1, raised against frog kainate-binding protein (26), reacted with the lower and higher molecular mass species in Western blots of purified eluates (Fig. 1, *lane B*) and synaptosomal membranes (Fig. 1, *lane C*). This suggests that the protein bands share a common epitope with the frog kainate-binding protein. To determine whether the higher molecular mass species were derived from the smaller species by disulfide cross-linking, we performed two-dimensional gel electrophoresis. The first dimension was an SDS gel run under nonreducing conditions, whereas the second was an SDS gel run under reducing conditions. A fraction of the 41-kDa and 45-kDa proteins could be derived from the 120-kDa band, as revealed in both silver-stained gels (Fig. 2B) and Western blots (Fig. 2A). This suggests that some of the kainate receptors from goldfish may be composed of at least two polypeptide subunits linked by disulfide bridges. Further evidence that the reduction of disulfide bonds may influence receptor behavior was obtained from [^{3}H]kainate binding assays of purified reconstituted receptor (Fig. 3). In the presence of 1 mM DTT, a reducing agent, [^{3}H]kainate binding was increased. Taken together, these data suggest that functional cysteine residues may influence kainate receptor binding and subunit structure.

The regional distribution of the 41-kDa and 45-kDa proteins was studied by dissecting specific brain regions and using Western blot analysis, with the KAR-B1 antibody, to determine the relative proportions of the two components. As shown in

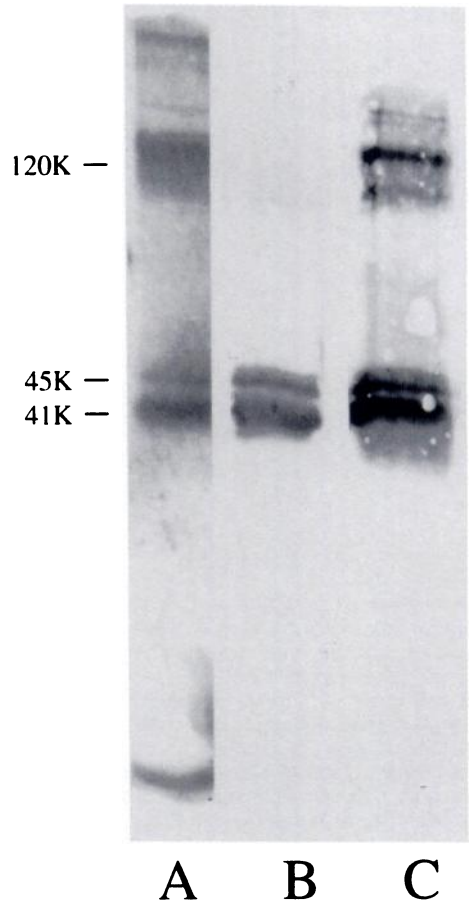

Fig. 1. SDS-polyacrylamide gel of affinity-purified receptors and immunoblots of affinity-purified receptors and synaptosomal membranes. *Lane A*, silver-stained 10% gel of purified receptor preparations; *lane B*, immunoblot of purified receptor preparations, using KAR-B1; *lane C*, immunoblot of synaptosomal membranes, using KAR-B1. KAR-B1 is a monoclonal antibody directed against purified frog kainate-binding protein (immunoblots were prepared as described in Experimental Procedures).

Fig. 4, the distribution varied with brain region, with the 45-kDa protein predominating in cerebrum, hypothalamus, optic tectum, spinal cord, and vagal lobe, the 41-kDa protein predominating in the lateral line lobe, and similar proportions of the two in the brainstem and cerebellum. This suggests that, although in some cases the two proteins can associate, they clearly exist as independent proteins in different brain regions.

To identify which of the protein bands contains the kainate binding site, purified preparations were incubated with [^{3}H]kainate and subjected to UV irradiation at 254 nm. This resulted in the cross-linking of [^{3}H]kainate to the 41-kDa protein (Fig. 5, *lane A*). Also consistently observed was very weak labeling of the 120-kDa band and, occasionally, of a band of unknown origin in the region of 60 kDa. This cross-linking was specifically and completely blocked in the presence of 300 μM unlabeled kainate or 600 μM CNQX (data not shown), a kainate antagonist. Photolabeling was significantly decreased by 300 μM GTPγS (Fig. 5, *lane B*). Because endogenous G protein was present in this preparation, the decreased incorporation of [^{3}H]kainate in the presence of GTPγS is probably due to a lower affinity for agonist caused by the uncoupling of activated G protein from the receptor, as observed for other G protein-linked receptors (reviewed in Ref. 23). In all preparations

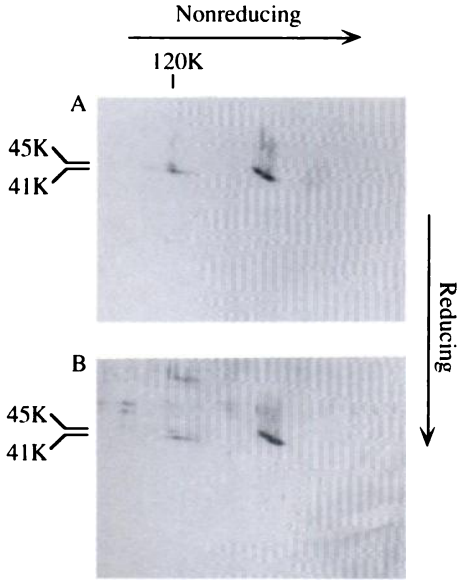

Fig. 2. Two-dimensional mapping of purified preparation. A, Immunoblot using KAR-B1; B, silver-stained 10% SDS-polyacrylamide gel. Proteins from a purified preparation were separated by SDS-polyacrylamide gel electrophoresis with reducing agent not present. An entire lane was excised and was subjected to SDS-polyacrylamide gel electrophoresis in the other direction, with 10% β-mercaptoethanol present in the sample buffer (immunoblots were prepared as described in Experimental Procedures). Spots on the diagonal represent proteins that migrate the same in the presence as in the absence of reducing agents. Spots below the diagonal represent proteins that migrate faster in the presence than in the absence of reducing agents.

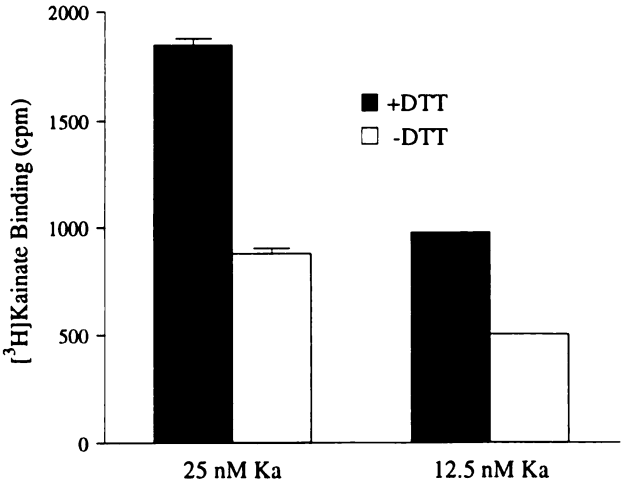

Fig. 3. Effects of DTT on equilibrium binding of [^{3}H]kainate. Purified/reconstituted preparations were preincubated for 1 hr in HMEN buffer, in the presence and absence of 2 mM DTT. Specific binding of 12.5 nM and 25 nM [^{3}H]kainate (*Ka*) to aliquots is shown. Assays were done as described in Experimental Procedures.

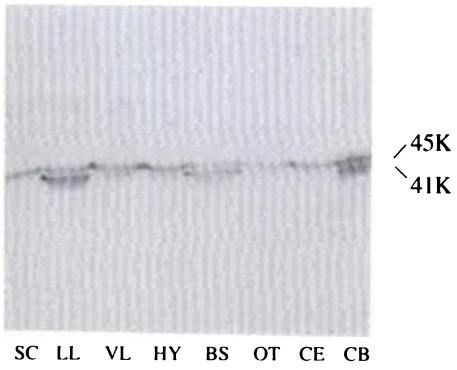

Fig. 4. Western blot analysis of proteins having epitopes recognized by the KAR-B1 antibody. Crude synaptosomal membranes from the brain regions indicated were solubilized in SDS and separated on 8% acrylamide gels. *SC*, spinal cord; *LL*, lateral line lobe; *VL*, vagal lobe; *HY*, hypothalamus; *BS*, brainstem; *OT*, optic tectum; *CE*, cerebrum; *CB*, cerebellum. In the case of the optic tectum, the eppendymal layer was removed before homogenization.

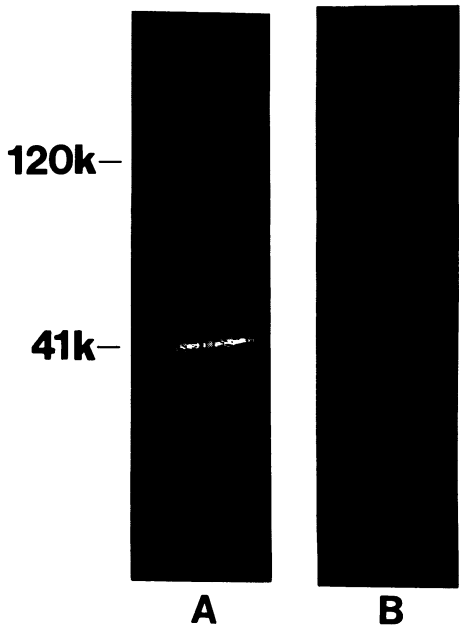

Fig. 5. Photoincorporation of [^{3}H]kainate by purified receptors. Photoaffinity labeling was performed in the absence (*lane A*) and presence (*lane B*) of 300 μM GTPγS. Shown are autoradiograms of 8% SDS gels of purified preparations. Preparations were affinity-purified in the absence of a GTPγS wash, to co-purify endogenous G proteins with receptor. Photoaffinity labeling was done as described in Experimental Procedures.

studied (four experiments), no [^{3}H]kainate incorporation into the 45-kDa protein was observed upon exposure to UV irradiation (254 nm).

As shown above, the 41- and 45-kDa proteins share a common epitope with the frog kainate-binding protein but can exist independently in different regions of the CNS. This suggests that both are structurally related to the frog kainate-binding protein. However, the question remains of whether these protein bands represent two distinct proteins or a single protein, covalently modified such that the mobility on SDS-polyacrylamide gels is affected. Potential covalent modifications could include factors such as partial proteolysis or differential glycosylation or phosphorylation. To probe the relationship between these polypeptides further, partial proteolytic digests of the 41- and 45-kDa proteins were compared. Fig. 6 shows the results of these experiments. Peptide mapping with *S. aureus* V8 protease revealed marked differences in the cleavage patterns, as shown on silver-stained SDS-polyacrylamide gels (Fig. 6, *lanes B* and *C*) and Western blots (Fig. 6, *lanes D* and *E*). Virtually no peptide bands of the same apparent molecular mass were in common. Furthermore, the monoclonal antibody KAR-B1 recognized five bands from the 41-kDa protein but only three from the 45-kDa protein (Fig. 6, *lanes D* and *E*), suggesting that the two parent proteins differ structurally (the 19-kDa band labeled in the 45-kDa digest is probably a contam-

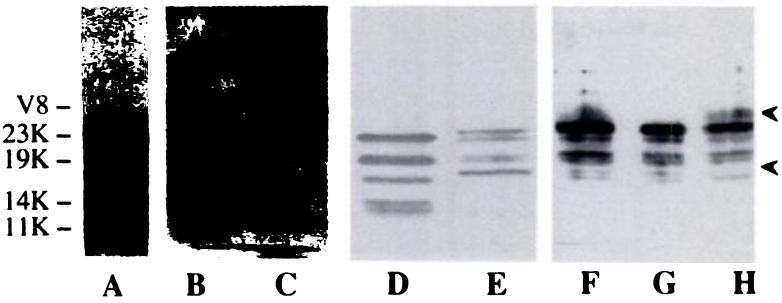

Fig. 6. Partial proteolytic mapping of the 41-kDa and 45-kDa proteins. Purified proteins were separated on 10% SDS gels. The bands were excised separately and subjected to electrophoresis on 12% SDS gels, in the presence of *S. aureus* V8 protease (*lanes A–E*) or chymotrypsin (*lanes F–H*), as described (28). *Lane A*, autoradiogram of a V8 protease peptide map of the 41-kDa band, photoaffinity labeled with [^{3}H]kainate before initial electrophoresis; *lanes B* and *C*, silver-stained gels of peptide maps of the 41-kDa (*lane B*) and 45 kDa (*lane C*) bands; *lanes D* and *E*, immunoblots of peptide maps of the 41-kDa (*lane D*) and 45-kDa (*lane E*) bands, using KAR-B1; *lanes F, G*, and *H*, silver-stained gels of peptide maps of the 41-kDa (*lane H*) and 45-kDa (*lane G*) bands and both bands together (*lane F*). The arrows indicate the peptide bands that differ between *lanes G* and *H*.

inant from the 41-kDa protein). Western blots of chymotryptic digests (Fig. 6, *lanes F, G*, and *H*) provided additional support for this conclusion. Although the patterns were more similar than the V8 digests, there were definite differences (Fig. 6, *arrows*).

The mobility of a protein on SDS-polyacrylamide gels can be altered by various types of covalent modifications. We investigated the possibility that the two protein bands represent different glycosylation states of the same protein. To deglycosylate purified preparations, aliquots were incubated with endoglycosidase F/*N*-glycosidase F. Fig. 7 shows a Western blot both in the absence and in the presence of these enzymes. A slight increase in the mobility of both proteins was observed and, as determined by the staining density, the 45-kDa protein appeared to have traveled slightly faster than the 41-kDa protein. This resulted in a closely spaced doublet at approximately 38–39 kDa (Fig. 7, *arrows*). Phosphorylation also has the potential to change the mobility of proteins on gels, generally reducing it. Therefore, the difference in the apparent migration of the 41- and 45-kDa polypeptides could be due to the phosphorylation state of each. Three purified preparations, incubated with potato acid phosphatase, showed no shift in mobility of either protein, as revealed on Western blots (data not shown). Taken together, these data indicate that the two protein bands probably do not represent different phosphorylated or glycosylated states of the same protein.

At least a portion of the [^{3}H]kainate binding site is contained in the 41-kDa polypeptide (see above and Fig. 5). To examine this site in greater detail, a photoaffinity-labeled 41-kDa band was excised from a SDS gel and subjected to peptide mapping using *S. aureus* V8 protease (Fig. 6, *lane A*), under the same conditions as for unlabeled protein (Fig. 6, *lane B*). Three cleavage products were strongly labeled, corresponding to the 23-, 14-, and 11-kDa fragments seen on the silver-stained gel (Fig. 6, *lane B*). Additionally, the 16-kDa band was labeled, although less intensely. Three of these fragments (23-, 16-, and 14-kDa) were also recognized by the KAR-B1 antibody (Fig. 6, *lane D*); however, this antibody does not appear to be directed to the [^{3}H]kainate binding site of the frog receptor (26).

We have obtained a partial amino acid sequence from the 23-kDa proteolytic cleavage fragment of the 41-kDa protein. The best alignment of this sequence with other published amino acid sequences of kainate/glutamate receptors is shown in Fig. 8. Seven to 10 residues of the 17 residues in this region of the goldfish 41-kDa protein are identical to those in the proteins that either are activated by kainate or bind kainate with high affinity. The metabotropic glutamate receptor, which is not activated by kainate, has no sequence homology with this segment (11). Interestingly, this region is proximal to the first putative membrane-spanning region and is, thus, an extracellular portion. Taken together, these data potentially identify a region of kainate binding. To date, we have not been able to

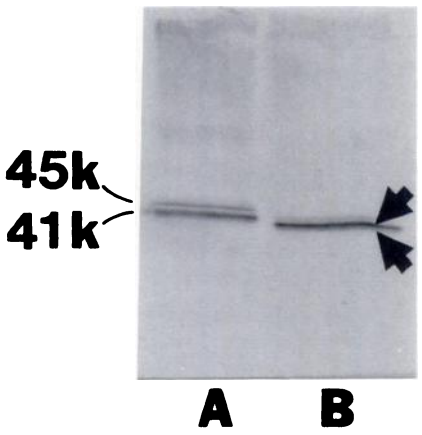

Fig. 7. Deglycosylation of purified proteins with endoglycosidase F/*N*-glycosidase F. Shown are Western blots of a 10% acrylamide SDS gel, using the KAR-B1 monoclonal antibody. *Lane A*, not treated with enzyme; *lane B*, treated with the enzyme mixture. *Arrows*, position of the new doublet formed with this treatment.

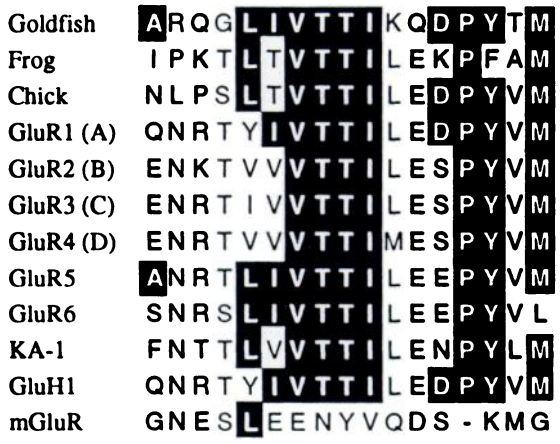

Fig. 8. Comparison of the partial sequence of the 41-kDa protein with other known non-NMDA glutamate receptors. The sequence positions shown for the non-NMDA glutamate receptors are as follows: frog, 20–36 (9); chick, 22–38 (10); GluR1 (A), 387–403 (4, 5); GluR2 (B), 390–406 (3, 5); GluR3 (C), 392–408 (3, 5); GluR4 (D), 392–408 (4, 5); GluR5, 415–431 (31); GluR6, 398–414 (7); KA-1, 394–410 (8); GluH1, 386–402 (6); and mGluR, 396–412 (32). The alignment of mGluR with the remainder of the sequences is presented as in the report of Masu *et al*. (32).

obtain sequences for the lower molecular mass proteolytic cleavage fragments, which may allow more precise determination of the [^{3}H]kainate binding site. We have also not obtained sequence data on the 45-kDa polypeptide.

Discussion

The existence of two polypeptides in our purified eluates after removal of G proteins can be explained in three possible ways; 1) the 41-kDa protein could be a partial proteolytic fragment of the 45-kDa protein, 2) the two bands could represent two variations of post-translational modification in a single protein, or 3) these bands could be separate proteins, products of different mRNAs. There are several points to consider in trying to discriminate between these possibilities. The two proteins seem to exist independently, in that they are differentially distributed in major brain regions. Peptide maps of the 41- and 45-kDa bands were generated and compared with each other. The difference between these maps suggests that the proteins may be structurally distinct but related. Proteolytic digestion with *S. aureus* V8 protease resulted in markedly distinct patterns, whereas chymotrypsin generated more related patterns; however, at no time was a polypeptide of 41 kDa derived from the 45-kDa band. These data suggest that these two polypeptides might differ either in amino acid sequence or by post-translational modification, although, as indicated by the chymotrypsin experiment, some degree of homology may be present. This concurs with the recognition of both bands by the same monoclonal antibody. Peptide sequencing clearly indicated that the 41-kDa protein has sequence homology with non-NMDA glutamate receptors; however, no sequence data could be obtained from the 45-kDa protein.

Although the two-dimensional gel electrophoresis results suggest that in some cases the 41-kDa and the 45-kDa proteins can be disulfide linked, the regional distribution of the two proteins, illustrated by the Western blot in Fig. 4, indicates that they do exist separately in different brain regions. In the brain regions that contain the highest level of kainate binding (cerebellum and lateral line lobe) (see Ref. 29), the 41-kDa protein predominates.

Post-translational modifications can affect the mobility of proteins on SDS gels. The two most common types of modifications that have been shown to cause mobility shifts are glycosylation and phosphorylation. Treatment with endoglycosidase F/*N*-glycosidase F, an enzyme mixture that cleaves at a wide variety of *N*-linked sugar moieties, did not convert the 45-kDa band into the 41-kDa band; however, it did generate a new doublet at 38/39 kDa, with the concomitant disappearance of the 41/45-kDa doublet. This suggests that, although both bands are *N*-glycosylated, they probably are not identical. The kainate receptor from chick brain has been shown to be a substrate for the cAMP-dependent protein kinase and, therefore, can become phosphorylated (30), suggesting that the doublet could be produced by differential phosphorylation. However, no difference in apparent molecular mass was seen in either band after treatment with potato acid phosphatase, suggesting that the 45-kDa protein probably does not represent a phosphorylated state of the 41-kDa band. Decreases in mobility on SDS gels due to phosphorylation can occur but are highly variable; therefore, further studies are needed to determine whether either purified protein is in a phosphorylated state. These data suggest that structural dissimilarity of the 41- and 45-kDa proteins is not likely to be due to a post-translational modification.

The results described above indicate that the 41-kDa protein probably is not a proteolytic fragment of the 45-kDa protein. Additional support for this conclusion is as follows. Very little variation in the ratio of the two polypeptides has been observed over the course of approximately 50 purifications from goldfish whole brain. The presence or absence of protease inhibitors during purification does not appear to affect the ratio. Upon storage at 4°, the 41-kDa protein does not accumulate at the expense of the 45-kDa protein. Furthermore, synaptic membranes, made on ice in the presence of a cocktail of protease inhibitors, display the same pattern of labeled bands in Western blots as do purified preparations. The 41-kDa and 45-kDa bands seem to be present at the same relative proportions in both preparations. Under the conditions used for membrane preparation, it is unlikely that proteolysis had occurred. Together, these data suggest that the two protein bands may represent related but distinct proteins, encoded by different mRNAs.

In our study, [^{3}H]kainate photoaffinity labeled only the 41-kDa protein. The lack of labeling of the 45-kDa protein could be due to either the absence of a kainate binding site or a lower efficiency of incorporation of [^{3}H]kainate into this protein. The latter could be explained by a lower affinity for [^{3}H]kainate of the higher molecular mass protein or by differences in the amino acid sequence. A saturating concentration (350 nM) of [^{3}H]kainate was used, in order to increase the probability of obtaining a strong signal. The binding site of the 45-kDa protein is unlikely to be cleaved proteolytically, because the apparent molecular mass of this protein is greater than that of the protein that is photoaffinity labeled. Significant kainate binding is observed in the spinal cord (29), where no 41-kDa protein was detected on immunoblots, suggesting the possibility that the 45-kDa protein may also bind kainate.

Many affinity-purified eluents contained not only 45-kDa and 41-kDa protein bands but also bands at approximately 115 kDa and 135 kDa. The higher molecular mass species were present under both reducing and nonreducing conditions, and all were recognized on Western blots. Immunoblots of synaptosomal preparations were very similar to those of purified eluents. Our data suggest that the 41-kDa and 45-kDa proteins are derived from the higher molecular mass species. Additionally, in photoaffinity labeling experiments in which the higher molecular mass proteins were present, both the 41-kDa protein and a higher molecular mass species were labeled (Fig. 5), suggesting that the 41-kDa band is present in both higher molecular mass species. Other antibodies raised against purified kainate receptors also recognize proteins with an approximate molecular mass of 100 kDa (26) in frog, chick, and rat brain. In frog, at least a portion of the higher molecular mass protein is composed of oligomers of the 48-kDa kainate-binding protein. However, in rat, reducing agents do not decrease the apparent molecular mass of a 99-kDa protein recognized by antibody (26). This is in agreement with the identification of a family of non-NMDA glutamate receptors in rat brain, having molecular masses of approximately 100 kDa (3–5). These are ion channels that are activated by kainate but apparently bind kainate with low affinity. In addition, a high affinity kainate-binding protein has been cloned from rat brain, with a predicted molecular mass of 105 kDa (8). Similar to the nonmammalian

receptors, the physiological response of this protein has not been defined. The carboxyl-terminal region of these mammalian proteins has approximately 30–40% sequence homology with the low molecular mass kainate-binding proteins. Given that some of the high molecular mass proteins in the present report are recognized by the antibody KAR-B1 and cannot be converted to a lower molecular mass species by reducing agents, a homolog of one of these larger molecular mass mammalian proteins may be present in goldfish brain. Also, the possibility cannot be excluded that the 41-kDa and 45-kDa polypeptides are derived from specific proteolytic cleavage of the higher molecular mass protein.

There have been several reports concerning both the purification and the cloning of the low molecular weight kainate receptors from nonmammalian species. To date, there has been little evidence of subunit structure or multiple receptors from the same species. A series of purified frog kainate receptors (M_r 48,000) have been separated by isoelectric focusing (26) and were probed by a series of antibodies, including KAR-B1, by Western blotting. Some slight variability in antibody recognition was observed, suggesting possible heterogeneity; however, V8-generated peptide maps of these proteins were indistinguishable, indicating a high degree of homology. The results might be explained by some type of post-translational modification; however, the presence of more then one protein cannot be ruled out. The evidence presented suggests the existence of at least two different kainate receptor polypeptides. We have previously shown that a kainate receptor from goldfish brain is coupled to a pertussis toxin-sensitive G protein (20) with which it can be co-purified (21). We have shown here that incorporation of [^{3}H]kainate with UV irradiation occurs in the 41-kDa but not the 45-kDa polypeptide. Labeling is decreased in the presence of GTPγS, providing direct evidence that the 41-kDa protein couples to G proteins. However, the significance of the 45-kDa protein and its potential role in the G protein-coupled pathway are unknown, although the possibility exists that, in some cases, it could serve as a subunit of the kainate-binding protein.

Acknowledgments

The authors would like to thank Dr. Gregory Weiland and Dr. Richard Cerione (Department of Pharmacology, Cornell University) for their helpful discussions. We are also indebted to Dr. Robert Wenthold (National Institutes of Health) for his helpful advice and for supplying the KAR-B1 antibody and to Dr. Andrew Bass (Section of Neurobiology and Behavior, Cornell University) for performing the dissections of specific brain regions.

References

1. Watkins, J. C., and R. H. Evans. Excitatory amino acid transmitters. *Annu. Rev. Pharmacol. Toxicol.* **21**:165–204 (1981).
2. Foster, A. C., and G. E. Fagg. Acidic amino acid binding sites in mammalian neuronal membranes: their characteristics and relationship to synaptic receptors. *Brain Res. Rev.* **7**:103–164 (1984).
3. Boulter, J., M. Hollmann, A. O'Shea-Greenfield, M. Hartley, E. Deneris, C. Maron, and S. Heinemann. Molecular cloning and functional expression of glutamate receptor subunit genes. *Science (Washington D. C.)* **249**:1033–1037 (1990).
4. Hollmann, M., A. O'Shea-Greenfield, S. W. Rogers, and S. Heinemann. Cloning by functional expression of a member of the glutamate receptor family. *Nature (Lond.)* **342**:643–648 (1989).
5. Keinänen, K., W. Wisden, B. Sommer, P. Werner, A. Herb, T. A. Verdoorn, B. Sakmann, and P. H. Seeburg. A family of AMPA-selective glutamate receptors. *Science (Washington D. C.)* **249**:556–560 (1990).
6. Puckett, C., C. M. Gomez, J. R. Korenberg, H. Tung, T. J. Meier, X. N. Chen, and L. Hood. Molecular cloning and chromosomal localization of one of the human glutamate receptor genes. *Proc. Natl. Acad. Sci. USA* **88**:7557–7561 (1991).
7. Egebjerg, J., B. Bettler, I. Hermans-Borgmeyer, and S. Heinemann. Cloning of a cDNA for a receptor subunit activated by kainate but not AMPA. *Nature (Lond.)* **351**:745–748 (1991).
8. Werner, P., M. Voigt, K. Keinänen, W. Wisden, and P. H. Seeburg. Cloning of a putative high-affinity kainate receptor expressed predominantly in hippocampal CA3 cells. *Nature (Lond.)* **351**:742–744 (1991).
9. Wada, K., C. J. Dechesne, S. Shimasaki, R. G. King, K. Kusano, A. Buonanno, D. R. Hampson, C. Banner, R. J. Wenthold, and Y. Nakatani. Sequence and expression of a frog brain complementary DNA encoding a kainate-binding protein. *Nature (Lond.)* **342**:684–689 (1989).
10. Gregor, P., I. Mano, I. Maoz, M. McKeown, and V. Teichberg. Molecular structure of the chick cerebellar kainate-binding subunit of a putative glutamate receptor. *Nature (Lond.)* **342**:689–692 (1989).
11. Tanabe, Y., M. Masu, T. Ishii, R. Shigemoto, and S. Nakanishi. A family of metabotropic glutamate receptors. *Neuron* **8**:169–179 (1992).
12. Coyle, J. T. Neurotoxic action of kainic acid. *J. Neurochem.* **41**:1–11 (1983).
13. Gallo, V., C. Giovannini, and G. Levi. Quisqualic acid modulates kainate responses in cultured cerebellar granule cells. *J. Neurochem.* **52**:10–16 (1989).
14. Garthwaite, J., E. Southam, and M. Anderton. A kainate receptor linked to nitric oxide synthesis from arginine. *J. Neurochem.* **53**:1952–1954 (1989).
15. Cornell-Bell, A. H., S. M. Finkbeiner, M. S. Cooper, and S. J. Smith. Glutamate induces calcium waves in cultured astrocytes: long-range glial signaling. *Science (Washington D. C.)* **247**:470–473 (1990).
16. Jensen, A. M., and S. Y. Chiu. Fluorescence measurement of changes in intracellular calcium induced by excitatory amino acids in cultured cortical astrocytes. *J. Neurosci.* **10**:1165–1175 (1990).
17. Murphy, S. N., and R. J. Miller. A glutamate receptor regulates Ca^{2+} mobilization in hippocampal neurons. *Proc. Natl. Acad. Sci. USA* **85**:8737–8741 (1988).
18. Rovira, C., M. Gho, and Y. Ben-Ari. Block of $GABA_b$-activated K^+ conductance by kainate and quisqualate in rat CA3 hippocampal pyramidal neurones. *Pflügers Arch.* **415**:471–478 (1990).
19. Ishida, A. T., and J. Neyton. Quisqualate and L-glutamate inhibit retinal horizontal-cell responses to kainate. *Proc. Natl. Acad. Sci. USA* **82**:1837–1841 (1985).
20. Willard, J. M., C. J. Ziegra, and R. E. Oswald. The interaction of a kainate receptor from goldfish brain with a pertussis toxin-sensitive GTP-binding protein. *J. Biol. Chem.* **266**:10196–10200 (1991).
21. Ziegra, C. J., J. M. Willard, and R. E. Oswald. Coupling of a purified goldfish brain kainate receptor with a pertussis toxin-sensitive G protein. *Proc. Natl. Acad. Sci. USA* **89**:4134–4138 (1992).
22. Hampson, D. R., and R. J. Wenthold. A kainic acid receptor from frog brain purified using domoic acid affinity chromatography. *J. Biol. Chem.* **263**:2500–2505 (1988).
23. Gilman, A. G. G proteins and dual control of adenylate cyclase. *Annu. Rev. Biochem.* **56**:615–649 (1987).
24. Gregor, P., N. Eshhar, A. Ortega, and V. Teichberg. Isolation, immunochemical characterization and localization of the kainate subclass of glutamate receptor from chick cerebellum. *EMBO J.* **7**:2673–2679 (1988).
25. Klein, A. U., B. Niederoest, K. H. Winterhalter, M. Cuénod, and P. Streit. A kainate binding protein in pigeon cerebellum: purification and localization by monoclonal antibody. *Neurosci. Lett.* **95**:359–364 (1988).
26. Hampson, D. R., K. D. Wheaton, C. J. Dechesne, and R. J. Wenthold. Identification and characterization of the ligand binding subunit of a kainic acid receptor using monoclonal antibodies and peptide mapping. *J. Biol. Chem.* **264**:13329–13335 (1989).
27. Cerione, R. A., J. W. Regan, H. Nakata, J. Codina, J. L. Benovic, P. Gierschik, R. L. Somers, A. M. Spiegel, L. Birnbaumer, R. J. Lefkowitz, and M. G. Caron. Functional reconstitution of the α_2-adrenergic receptor with guanine nucleotide regulatory proteins in phospholipid vesicles. *J. Biol. Chem.* **261**:3901–3909 (1986).
28. Cleveland, D. W., S. G. Fischer, M. W. Kirschner, and U. K. Laemmli. Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. *J. Biol. Chem.* **252**:1102–1106 (1977).
29. Ziegra, C. J., R. E. Oswald, and A. H. Bass. [^{3}H]Kainate localization in goldfish brain: receptor autoradiography and membrane binding. *Brain Res.* **527**:308–317 (1990).
30. Ortega, A., and V. I. Teichberg. Phosphorylation of the 49-kDa putative subunit of the chick cerebellar kainate receptor and its regulation by kainatergic ligands. *J. Biol. Chem.* **265**:21404–21406 (1990).
31. Bettler, B., J. Boulter, I. Hermans-Borgmeyer, A. O'Shea-Greenfield, E. S. Deneris, C. Moll, U. Borgmeyer, M. Hollman, and S. Heinemann. Cloning of a novel glutamate receptor subunit, GluR5: expression in the nervous system during development. *Neuron* **5**:583–595 (1990).
32. Masu, M., Y. Tanabe, K. Tsuchida, R. Shigemoto, and S. Nakanishi. Sequence and expression of a metabotropic glutamate receptor. *Nature (Lond.)* **349**:760–765 (1991).

Send reprint requests to: Robert E. Oswald, Department of Pharmacology, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853.