Solubilization, Partial Purification, and Reconstitution of Glutamate- and *N*-Methyl-D-aspartate-Activated Cation Channels from Brain Synaptic Membranes†

A. M. Ly and E. K. Michaelis*

Department of Pharmacology and Toxicology and Center for Biomedical Research, University of Kansas, Lawrence, Kansas 66045

Received October 23, 1990; Revised Manuscript Received December 31, 1990

ABSTRACT: L-Glutamate-activated cation channel proteins from rat brain synaptic membranes were solubilized, partially purified, and reconstituted into liposomes. Optimal conditions for solubilization and reconstitution included treatment of the membranes with nonionic detergents in the presence of neutral phospholipids plus glycerol. The affinity batch chromatography procedure described previously [Chen et al. (1988) *J. Biol. Chem. 263*, 417–427] was used to obtain a fraction enriched in glutamate-binding proteins. Quench-flow procedures were developed to characterize the rapid kinetics of ion flux induced by receptor agonists. [^{14}C]Methylamine, a cation that permeates through the open channel of both vertebrate and invertebrate glutamate receptors, was used to measure the activity of glutamate receptor–ion channel complexes in reconstituted liposomes. L-Glutamate caused an increase in the rate of [^{14}C]methylamine influx into liposomes reconstituted with either solubilized membrane proteins or partially purified glutamate-binding proteins. The increase in methylamine influx was dependent on the concentration of L-glutamic acid with an estimated K_{act} for L-glutamate equal to 0.2 μM for synaptic membrane proteins and 0.32 μM for purified proteins. Of the major glutamate receptor agonists, only *N*-methyl-D-aspartate activated cation fluxes in liposomes reconstitued with glutamate-binding proteins. Glutamate-activated methylamine flux was completely inhibited by the *N*-methyl-D-aspartate receptor antagonist 2-amino-5-phosphonopentanoic acid. In liposomes reconstituted with glutamate-binding proteins, *N*-methyl-D-aspartate- or glutamate-induced influx of Na^+ led to a transient increase in the influx of the lipid-permeable anion probe $S^{14}CN^-$. Electrophoretic analysis of partially purified proteins reconstituted in liposomes indicated enrichment of several bands, the most prominent being those of molecular size equal to ~69, 60, 35, and 25 kDa. Antibodies raised against the purified 71- and 63-kDa glutamate-binding proteins reacted strongly with the ~69-kDa band of reconstituted proteins and markedly decreased the initial rate of glutamate-activated cation flux. These results indicate the functional reconstitution of *N*-methyl-D-aspartate-sensitive glutamate receptors and the role of the ~69-kDa protein in the function of these ion channels.

The case for L-glutamic acid as an excitatory neurotransmitter in the mammalian central nervous system has been reviewed extensively [e.g., see Curtis and Johnston (1974), Krnjevic, (1974), Watkins and Evans (1981), Cotman and Iversen (1987), and Cotman et al., (1988)]. L-Glutamic acid released from nerve terminals diffuses across the synaptic cleft and reaches postsynaptic receptors. Activation of these receptors leads to one of two types of responses, either activation of phospholipase C and metabolism of phosphatidylinositol (Recasens et al., 1988; Sladeczek et al., 1988; Sugiyama et al., 1989) or increases in neuronal membrane Na^+, K^+, and Ca^{2+} permeability and subsequent neuronal excitation [e.g., see Nowak et al. (1984), MacDermott et al. (1986), Cull-Candy and Usowicz (1987), and Jahr and Stevens (1987)].

A further subclassification of the excitatory amino acid receptors linked to ion channels on the basis of the sensitivity of these receptors to specific agonists was first proposed by Watkins and colleagues (Watkins & Evans, 1981) and further amplified recently [e.g., see Watkins et al. (1990)]. These receptors are subclassified to the three major types activated by α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA),[1] an analogue of quisqualic acid, by kainic acid and by *N*-methyl-D-aspartate (NMDA). The best characterized class of ion channel linked receptors with respect to agonist and antagonist effects is the NMDA receptor for which very selective antagonists exist, such as 2-amino-5-phosphonopentanoic, 2-amino-7-phosphonoheptanoic acid, and 3-carboxypiperazin-4-yl-phosphonic acid.

In electrophysiological studies of single-channel characteristics of these three subtypes of glutamate receptors, it was shown that all types of receptors activate ion channels that exhibit the same multiple conductance states (Cull-Candy & Usowicz, 1987; Jahr & Stevens, 1987). In recent studies, it was also demonstrated that the NMDA and AMPA receptors coexist in the same neuronal synapses (Bekkers & Stevens, 1989). These observations have led to the suggestion either that all receptor recognition macromolecules are linked to one type of ion channel complex or that different pairs of recognition macromolecules activate the same ion channel complex through interactions with distinct recognition subunits (Cull-Candy & Usowicz, 1987). Further resolution of the

† This research was supported by grants DAAL 03-86-K-0086 and DAAL 03-88-K0017 from the ARO, DAMD 17-86-G-6038 from the U.S. Army Medical Research Command, and AA 04732 from NIAAA.

* Address correspondence to this author at the Center for Biomedical Research, University of Kansas, 2099 Constant Ave., Campus West, Lawrence, KS 66046.

[1] Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; DTT, dithiothreitol; EDTA, ethylene diaminetetraacetic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; NMDA, *N*-methyl-D-aspartic acid; PE, phosphatidylethanolamine; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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structure of excitatory amino acid receptors can only come from dissection of the protein composition of each receptor subtype.

The structure of a kainate receptor was recently elucidated by the cloning of the cDNA for a kainate receptor protein and the expression of the receptor–ion channel complex in frog oocytes following injection of mRNA formed from transcription of the cDNA (Hollman et al., 1989). The structure of the kainate receptor protein inferred from the cDNA represents a protein of molecular size equal to approximately 100 kDa. NMDA did not activate the kainate receptor–ion channel expressed in frog oocytes following injections of the oocytes with mRNA derived from the cloned cDNA for the 100-kDa protein. Quisqualate and AMPA, however, activate this receptor–ion channel protein expressed in mammalian cells and frog oocytes (Keinanen et al., 1990; Boulter et al., 1990). These results would indicate that NMDA receptors may have a different protein composition from the subtype of receptor which is a kainate/AMPA receptor–ion channel.

The structure of the NMDA receptor–ion channel complex isolated from rat brain neuronal membranes was recently reported to contain four protein subunits with molecular weights (M_r) of 67000, 57000, 46000, and 33000 (Ikin et al., 1990). We recently isolated from rat brain synaptic membranes a 58-kDa protein which has ligand-binding sites for the selective antagonists of NMDA receptors, the aminophosphonocarboxylic acids (Cunningham & Michaelis, 1990). This 58-kDa protein has the characteristics of a receptor-associated, antagonist-binding subunit and may correspond to the 57-kDa protein in the complex of the NMDA receptor proteins isolated by Ikin and colleagues (Ikin et al., 1990). We have also reported previously on the isolation and characterization of two glutamate-binding proteins from rat brain synaptic membranes with estimated molecular sizes equal to 71 and 63 kDa. Although the ligand selectivity for the binding sites of these proteins is not commensurate with that of NMDA receptors, it is possible that these proteins are structurally related to the 67-kDa protein subunit of the NMDA receptor complex isolated by Ikin et al. (1990).

An understanding of the structure and regulation of glutamate receptor complexes in neuronal membranes will come either from successful expression of receptor proteins through recombinant DNA techniques or through the isolation and functional reconstitution of proteins that are associated with these receptor–ion channel complexes. Great success was achieved in the definition and characterization of the nicotinic acetylcholine receptor–ion channel complex by the approach of protein isolation and functional reconstitution into liposomes or planar lipid bilayers [e.g., see Michaelson and Raftery (1974), Huganir and Racker (1980), and Suarez-Isla et al. (1983)]. Functional reconstitution of glutamate-activated ion channel proteins into liposomes or other lipid bilayers has not been described by any investigators. The studies reported in this paper represent the approaches that we pursued in order to determine the parameters that should be used for solubilization of synaptic membrane proteins and functional reconstitution of glutamate-activated ion channel proteins. We also describe the development of rapid kinetic procedures for the measurement of glutamate-activated cation fluxes and the reconstitution into liposomes of a partially purified preparation of glutamate-binding proteins which exhibited characteristics of L-glutamate-sensitive and NMDA-sensitive ion channels.

Materials and Methods

The source of [^{14}C]methylamine (48 mCi/mmol) was New England Nuclear, ^{22}NaCl and KS^{14}CN (50 mCi/mmol) were from Amersham/Searle Corp., and L-[^{3}H]glutamate (20 Ci/mmol) was from ICN Biomedicals. Dog brain phosphatidylethanolamine (PE),[1] cholesterol, and *n*-octyl glucoside were purchased from Sigma Chemical Co. Bio-Beads SM2 were obtained from Bio-Rad. Purified Triton X-100 was from Pierce. All other chemicals used were obtained from the sources described previosuly (Chen et al., 1988).

Preparation of Synaptic Membranes, Solubilization of Membrane Proteins, and Partial Purification of Glutamate-Binding Proteins. Synaptic membranes were isolated from four to eight brains of adult male Sprague-Dawley rats as described in Chen et al. (1988). Rat brain tissue was homogenized in the presence of six protease inhibitors (pepstatin, benzamide, phenylmethanesulfonyl fluoride, ϵ-aminocaproic acid, benzamidine hydrochloride, and EGTA) that were at the concentrations we have previously used (Chen et al., 1988). The synaptic membranes were resuspended in 20 mL of buffer containing 0.25 M sucrose, 20 mM potassium acetate, 10 mM Tris–sulfate, 0.1 mM dithiothreitol (DTT), 0.1 mM EDTA, 0.5 mg/mL PE, and 10% glycerol, pH 8.0. To this suspension was added an equal volume of the same buffer that contained either 4% (v/v) Triton X-100 or 4% (w/v) *n*-octyl glucoside. Triton X-100 solubilization was employed in all experiments that included further purification of glutamate-binding proteins, whereas *n*-octyl glucoside was the detergent used in studies of synaptic membrane protein solubilization and reconstitution. Solubilization was achieved by gentle hand-homogenization and stirring for 45 min at 0 °C. The detergent-treated preparation was centrifuged at 40000*g* for 40 min and the supernatant collected.

Further purification of L-glutamate-binding proteins from the solubilized membrane extract was carried out by affinity batch separation on glass fiber with coreticulated L-glutamate (Chen et al., 1988). After overnight incubation of the soluble extract with the glass fiber matrix, the non-glutamate-binding proteins were eluted with 200–300 mL of solubilization buffer that contained 0.1% Triton X-100, and the glutamate-binding proteins were eluted by two consecutive 4-h incubations at 4 °C with 25 mL of the same buffer solution containing 0.1% *n*-octyl glucoside and 5 mM L-glutamate. The eluates and washes were combined and dialyzed extensively against buffer that contained 20 mM potassium acetate, 10 mM Tris–sulfate, 0.1 mM DTT, 0.1 mM EDTA, and 10% glycerol, pH 8.0, but did not contain detergent or glutamate. The samples were concentrated by dialysis against poly(vinylpyrrolidone) (M_r 40000).

Reconstitution of Solubilized Synaptic Membrane Proteins or Partially Purified Glutamate-Binding Proteins into Liposomes. Reconstitution into liposomes of synaptic membrane proteins that were solubilized in *n*-octyl glucoside was initiated by adding a mixture of PE/cholesterol (4:1 weight ratio) in solubilization buffer to a final concentration of 10 mg of lipid/mL of solution. Following overnight dialysis of the suspension against potassium acetate/EDTA/DTT/Tris-sulfate buffer, the suspension was concentrated to 5–10 mL by dialysis against poly(vinylpyrrolidone). This was followed by reconstitution of proteins into liposomes after extraction of detergent through incubation (15 min at 4 °C) with polystyrene beads (Bio-Beads) in capped columns. The eluate was centrifuged at 300000*g* for 1 h to collect the liposomes formed.

The reconstitution of partially purified glutamate-binding proteins into liposomes was performed according to the following procedure. The concentrated samples of glutamate-binding protein (10–20 mL) were added to 60–100 mg of a washed and dried lipid mixture of PE and cholesterol (4:1

weight ratio). This was followed by reconstitution of proteins into liposomes following detergent extraction through incubation with polystyrene beads. The eluate was centrifuged at 300000g for 1 h to collect the pelleted liposomes.

Protein Determination. The amount of protein in particulate fractions was determined by the procedure of Peterson (1977), whereas that in solubilized and liposome-reconstituted samples was measured by the fluorescence procedure of Neuhoff et al. (1979).

Determination of Rapid Kinetics of [^{14}C]Methylamine and $S^{14}CN^-$ Influx. A Durrum Multi-Mixing system was used to perform assays of the rapid kinetics of [^{14}C]methylamine and $S^{14}CN^-$ influx into proteoliposomes. The rapid-mix–quench technique was employed. A liposome suspension in 10 mM Tris–sulfate/0.1 mM EDTA buffer, pH 7.4, was loaded into one of the syringes. The incubation medium consisting of 0.2 mM EDTA, 0.1 mM $MgSO_4$, 2 μM glycine, and 10 mM Tris–sulfate, pH 7.4, that contained either 10 mM methylamine ([^{14}C]methylamine, 5 μCi/mL) or 25 mM Na_2SO_4 and 1 mM KSCN (K$S^{14}CN$, 2 μCi/mL), was loaded in the other syringe. The liposome suspension and the incubation solution were delivered in equal volumes from the two reagent syringes. The quench solution contained 0.5 mM 2-amino-5-phosphonopentanoic acid (or 1 mM 2-amino-3 phosphonopropionic acid), 2 mM L-glutamate diethyl ester, and 0.5 mM $MgSO_4$ in 10 mM Tris–sulfate buffer, pH 7.4, and was delivered from the third syringe. Appropriate volumes were used in order to maintain the ratios of the solutions equal to 1:1:8.3, respectively, and in order to collect 200 μL of the quenched reaction product. The quenched reaction volume was collected in a calibrated syringe, the volume noted, and the sample immediately filtered through 0.22-μm Millipore filters. The filters were washed with 3 mL of ice-cold buffer that contained 1 mM $MgSO_4$, 0.1 mM EDTA, and 10 mM Tris–sulfate, pH 7.4. Dissolution of filters and measurement of trapped radioactivity were conducted as described previously (Chang & Michaelis, 1980). Background ion flux was defined as the uptake in the absence of any agonists acting on L-glutamate receptors. The instrument was precalibrated by following the rate of hydrolysis of dinitrophenyl acetate by KOH according to the procedures recommended by the manufacturer, and the reaction times were continuously monitored by a computer through digital conversion of signals from the movement of the pneumatic drive.

Antibodies against the 71- and 63-kDa Proteins. Highly purified preparations of the 71- and 63-kDa proteins were used to immunize rabbits in order to raise antibodies against these glutamate-binding proteins (Eaton et al., 1990). The immune and preimmune sera previously collected and characterized for their reactivity against these two proteins (Eaton et al., 1990) were used in immunoblot staining of proteins electrotransferred to nitrocellulose filters and in isolation of IgG that was used in ion flux studies. The purification of IgG by precipitation with ammonium sulfate and chromatographic separation on DEAE-Sephadex was performed as described previously (Eaton et al., 1990). The purified IgG fractions were used immediately after purification. The purity of the IgG fractions was determined by polyacrylamide gel electrophoresis under denaturing conditions.

Polyacrylamide Gel Electrophoresis and Immunoblot Staining. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed as described previously (Chen et al., 1988). Either the gels were stained with silver nitrate (Wray et al., 1981) or the separated proteins in the gels were electrotransferred onto nitrocellulose sheets. Proteins transferred to nitrocellulose filters were stained with the colloidal gold preparation Aurodye according to the specifications of the manufacturer and as described previously (Chen et al., 1988).

Immunochemical staining of transferred proteins was performed according to the procedure of Blake and colleagues (Blake et al., 1984) using alkaline phosphatase conjugates of goat anti-rabbit antibodies (1:1000 dilution) and 5-bromo-4-chloro-3-indolyl phosphate together with nitroblue tetrazolium (blue reaction product). The nitrocellulose filter following electrotransfer of proteins was washed with phosphate-buffered saline that contained 0.5% Tween-20 and incubated with 3% (w/v) gelatin in phosphate-buffered saline for 1 h at room temperature to block the remaining protein-binding sites. The blocked membrane was incubated with anti-glutamate-binding protein antiserum (1:1000 dilution) in 50 mL of phosphate-buffered saline containing 1% (w/v) gelatin for a minimum of 4 h at 30 °C. The alkaline phosphatase reaction was developed in 0.1 M Tris-HCl buffer, pH 9.8, that contained 4 mM $MgCl_2$, nitroblue tetrazolium, and the substrate for the enzyme (Blake et al., 1984; Chen et al., 1988).

Data Analysis. All ion flux kinetic data were analyzed by computer-assisted, nonlinear, least-squares approximation to a first-order rate equation for flux with an offset value at zero time:

$$I_t = I_\infty(1 - e^{-Jt}) + A_0$$

The I terms represent specific ion flux in moles per milligram of protein in liposomes at time t (I_t) and at equilibrium (I_∞), A_0 is the estimated amount of ion associated with the liposomes at time zero, and J is a rate coefficient for the overall reaction including ligand binding to receptor proteins, activation of the ion channel, ion permeation through the channel, and possible time-dependent inactivation of the ion channel.

Results

Glutamate-Activated Ion Fluxes into Proteoliposomes Formed with Synaptic Membrane Proteins. When synaptic membranes were extracted with a buffer that contained 2% (v/v) Triton X-100, 10% (v/v) glycerol, and 0.5 mg/mL PE, an average of 83.2% (±4.3, SEM, n = 7 experiments) of the membrane proteins were solubilized. If n-octyl glucoside was used in place of the Triton X-100, an average of 50% (±10.6, SEM, n = 4 experiments) of membrane proteins were obtained in the solubilized fraction. In all experiments to be presented, the synaptic membranes were solubilized in Triton X-100 when the soluble extract was to be used for further protein purification. Otherwise, the membranes were solubilized in n-octyl glucoside, and PE/cholesterol (4:1) was added in a solution that contained the same detergent. The mixture was dialyzed and passed over Bio-Beads to remove the detergent and allow for the reconstitution of membrane proteins into liposomes. This method led to the formation of unilamellar liposomes observed under electron microscopy and to the incorporation of 68.1% (±8.2, SEM, n = 4 experiments) of the solubilized proteins into the liposomes.

Preliminary experiments were performed by using a manual procedure to measure ^{22}Na fluxes into liposomes during incubation periods ranging from 2 to 300 s. The measurement of ^{22}Na fluxes was used to select the optimal conditions for reconstitution of synaptic membrane proteins. These conditions were found to consist of solubilization of synaptic membrane proteins in the presence of nonionic detergents, the use of neutral phospholipids and cholesterol, and the employment of 10% glycerol during solubilization and subsequent chromatographic separation of glutamate-binding proteins. The

permeability to ^{22}Na of liposomes reconstituted with membrane proteins was 8–10 times greater than the permeability of liposomes formed under identical conditions but without any proteins incorporated. The increased permeability described above was evident under two different procedures for measuring ^{22}Na fluxes, gradient-dependent ^{22}Na flux and isotopic exchange flux according to the method of Garty et al. (1983). This is not an unusual observation since even phospholipid liposomes reconstituted with nonchannel proteins have much greater permeability to ions such as Na^+ when compared with liposomes formed without proteins (Papahadjopoulos et al., 1973). The addition of 8 μM L-glutamate caused an increase in the rate of $^{22}Na^+$ influx above background (data not shown).

The rate of ^{22}Na flux measured by this manual technique was slow and would be difficult to relate to receptor-mediated ion channel openings that occur in the range of millisecond time periods. Furthermore, since synaptic membranes also contain L-glutamate transport carriers which are known to cotransport Na^+ and L-glutamate (Kanner & Sharon, 1978), measurements of glutamate activation of $^{22}Na^+$ flux could be the result of both L-glutamate-induced openings of Na^+ channels as well as ^{22}Na cotransport with L-glutamate into the reconstituted liposomes. In order to avoid the contribution of transport systems to the ion fluxes detected in reconstituted preparations, we measured the glutamate activation of influx into liposomes of an ion, methylamine, which does not support L-glutamate transport and does not permeate voltage-gated ion channels very well, but has high permeability through quisqualate and L-glutamate postsynaptic receptors in locust muscle (Anwyl, 1977), and through NMDA and kainate receptors in chick brain neurons (Vyklicky et al., 1988).

In order to estimate the more rapid events of ion channel activation, we employed the quench-flow, rapid kinetics procedures described under Materials and Methods. All [^{14}C]-methylamine flux assays were performed in the presence of submicromolar concentrations of free magnesium ions buffered by EDTA in order to avoid inhibition of the NMDA receptors produced by Mg^{2+} (Nowak et al., 1984). In addition, the incubation media contained 1 μM glycine, final concentration, because glycine functions as an allosteric activator of NMDA receptors (Johnson & Ascher, 1987). Under these conditions, all three types of L-glutamate-activated receptors should have been responsive to the respective agonists. An additional feature of the rapid kinetics flux assays was the use of a quench solution to stop the further flux of ions at the termination point of the assay. This was accomplished through the use of high concentrations of the quisqualate receptor antagonist, L-glutamate diethyl ester, and of either one of the NMDA receptor antagonists, 2-amino-5-phosphonopentanoic acid or 2-amino-3-phosphonopropionic acid, together with elevated Mg^{2+} concentrations in the quench solution. The effects of these agents on glutamate-activated ion flux will be described later, but in general, 2-amino-5-phosphonopentanoic acid was the most effective inhibitor when introduced at the concentration used in the quench solution.

As shown in Figure 1A, the rate of background flux of [^{14}C]methylamine into liposomes reconstituted with membrane proteins was constant over the 100–800-ms reaction period. L-Glutamate at 5 μM final concentration brought about rapid enhancement of [^{14}C]methylamine flux into the reconstituted proteoliposomes. The cation flux in the presence of 5 μM L-glutamate was significantly greater than background flux, and the stimulation of cation flux appeared to be complete within the period of 200 ms (Figure 1A). The rate of ion flux into these liposomes during the late reaction period of 200–800

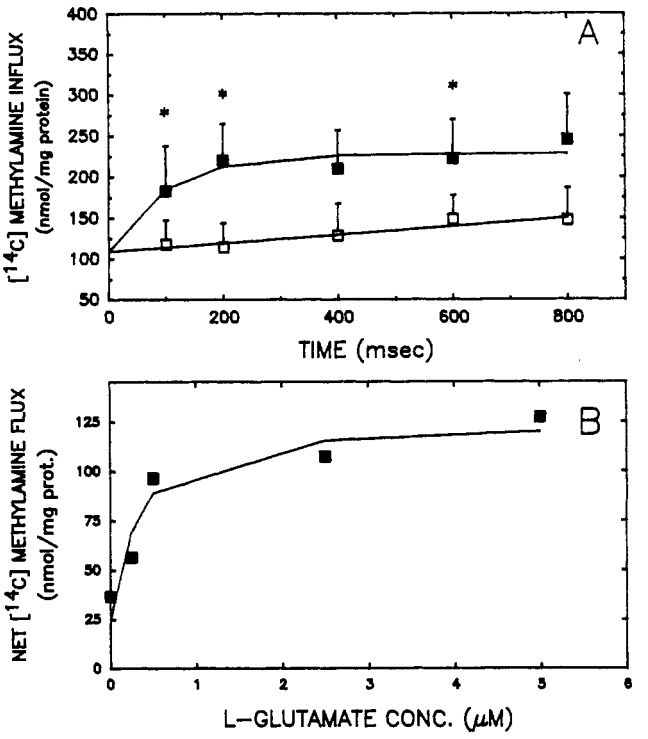

FIGURE 1: L-Glutamate activation of [^{14}C]methylamine flux into liposomes reconstituted with solubilized synaptic membrane proteins. (A) Rapid kinetics of [^{14}C]methylamine flux were determined in the absence (□) and in the presence of 5 μM L-glutamate (■). Membrane protein solubilization in *n*-octyl glucoside, reconstitution of solubilized proteins in PE/cholesterol liposomes, and rapid kinetic, quench-flow assays of [^{14}C]methylamine flux were performed as described under Materials and Methods. The data shown are the mean (±SEM) from five separate solubilization and reconstitution experiments. The curve drawn through the values for L-glutamate-enhanced [^{14}C]methylamine flux represents the best estimate for a pseudo-first-order rate equation with offset values as described under Materials and Methods. Background flux could not be fitted to a first-order rate equation; therefore, only a regression line is shown. The rate coefficient is $k = 9.4\ s^{-1}$, and maximal net flux above the offset value is $I_\infty = 162$ nmol/mg. Values for L-glutamate-induced cation flux that are significantly different from background flux are indicated (asterisk, $p \leq 0.05$ for one-tailed *t*-test statistic, 8 df). (B) Concentration-dependent enhancement by L-glutamate of [^{14}C]methylamine flux. Rapid kinetic assays of L-glutamate-induced [^{14}C]methylamine flux were performed as described above. The data for [^{14}C]methylamine influx into liposomes measured at 100-ms incubation in the absence of L-glutamate were subtracted from those determined in the presence of the concentrations of L-glutamate shown, i.e., net methylamine influx was calculated. The values shown are the mean net influx at 100 ms from four solubilization and reconstitution experiments. The curve drawn represents the best fit to the Michaelis-Menten type of equation with the following constants: $K_{act} = 0.2$ μM and $I_{max} = 125$ nmol/mg of proteins.

ms was not greater when measured in the presence of 5 μM L-glutamate than the constant rate of methylamine flux observed in liposomes incubated in the absence of L-glutamate. Assuming a pseudo-first-order rate of reaction for L-glutamate-induced [^{14}C]methylamine flux, the rate coefficient for this flux was estimated to be 9.4 s^{-1}, and I_∞ was 162 nmol/mg of protein (Figure 1A).

The activation of [^{14}C]methylamine flux into liposomes reconstituted with membrane proteins was dependent on the concentration of L-glutamate employed in these assays (Figure 1B). The estimated K_{act} for L-glutamate activation of [^{14}C]methylamine flux determined at 100 ms was 0.2 μM, and the maximal net flux at 100 ms was 125 nmol/mg of protein. There was no evidence of cooperativity in the activation of [^{14}C]methylamine flux by L-glutamate. The estimated K_{act} for L-glutamate-induced [^{14}C]methylamine flux is an order

of magnitude smaller than the K_{act} for L-glutamate transport carriers in synaptic membranes which is approximately equal to 2 μM (Kanner & Sharon, 1978a; Michaelis et al., 1982). The K_{act} for glutamate enhancement of cation flux is nearly identical with the K_D for L-[^{3}H]glutamate binding to synaptic membrane sites considered to be related to glutamate receptors [e.g., see Foster and Fagg (1984), Fagg and Matus (1984), and Monaghan et al. (1985)]. It appeared, therefore, that the glutamate activation of [^{14}C]methylamine flux was related to interaction of glutamate with membrane receptors.

The fact that L-glutamate transport into liposomes did not occur under the conditions of rapid kinetic measurements of [^{14}C]methylamine fluxes was also demonstrated directly. Assays were performed with reconstituted liposomes that were incubated with 0.5 and 1.0 μM L-[^{3}H]glutamate in the presence of 5 mM methylamine, final concentration. L-[^{3}H]Glutamate transport into the reconstituted liposomes during the 100–800-ms incubation period in the presence of methylamine was not greater than the background binding or entrapment measured in the presence of 12.5 mM K_2SO_4 (data not shown). The amount of L-[^{3}H]glutamate associated with liposomes that were incubated in the presence of 12.5 mM K_2SO_4 was considered background binding or entrapment because an inward-directed K^+ gradient across synaptic membranes does not support carrier-mediated L-glutamate transport into membrane vesicles (Kanner & Sharon, 1978a; Michaelis et al., 1982).

[^{14}C]Methylamine Influx into Proteoliposomes Reconstituted with Partially Purified Glutamate-Binding Proteins. One of the goals of these investigations was to determine whether a fraction of glutamate-binding proteins obtained following affinity chromatography of solubilized synaptic membrane proteins on L-glutamate-derivatized matrices had properties related to glutamate-activated ion channels. If such activity could be demonstrated in this partially purified protein fraction, then a more complete characterization of the proteins that form some of these channels might be more easily achieved. As is shown in Figure 2A, 5 μM L-glutamate caused an increase in [^{14}C]methylamine flux into liposomes reconstituted with glutamate-binding proteins. [^{14}C]Methylamine flux produced by L-glutamate was significantly greater than background during the 200–600-ms reaction period (Figure 2A). The liposomes reconstituted with partially purified glutamate-binding proteins also exhibited considerably higher background flux of [^{14}C]methylamine than that observed in liposomes reconstituted with membrane proteins (Figures 1A and 2A). This might be an indication either that there were more channels permeable to methylamine in the liposomes with the binding proteins or that the isolated protein fractions contained high amounts of associated detergents that were not completely removed by dialysis and Bio-Bead extraction.

The estimated rate coefficient for [^{14}C]methylamine flux in the presence of 5 μM L-glutamate added to the incubation medium was 8.6 s^{-1}, and I_∞ was 1173 nmol/mg of protein. The rate coefficient for L-glutamate-activated [^{14}C]methylamine flux in liposomes reconstituted with the binding protein fraction was nearly identical with that determined for proteoliposomes reconstituted with membrane proteins. However, the I_∞ was approximately 7 times that determined for liposomes reconstituted with synaptic membrane proteins. Analysis of the net [^{14}C]methylamine flux at 100-ms reaction times activated by increasing L-glutamate concentrations revealed a K_{act} for L-glutamate equal to 0.32 μM (Figure 2B). This estimated value of K_{act} for [^{14}C]methylamine flux into liposomes reconstituted with glutamate-binding proteins was nearly identical with the

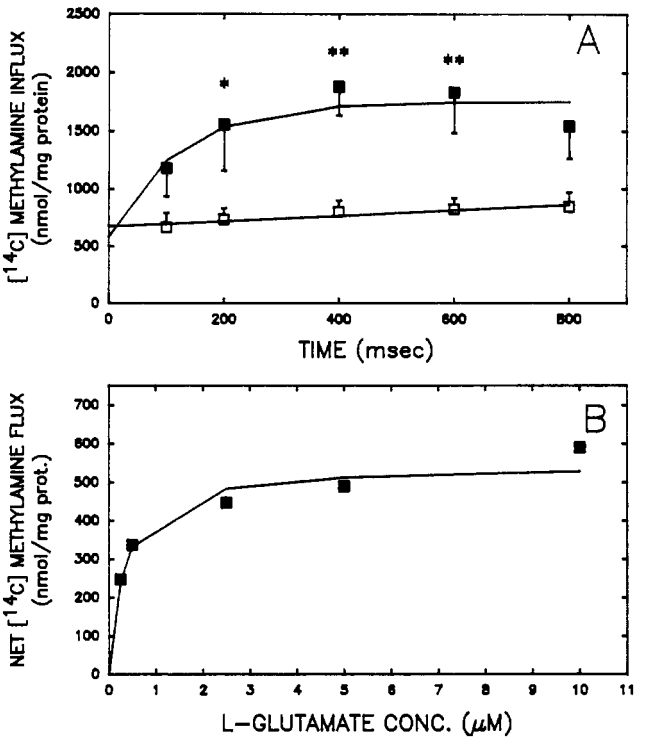

FIGURE 2: L-Glutamate activation of [^{14}C]methylamine flux into liposomes reconstituted with partially purified glutamate-binding proteins. (A) Rapid kinetics of [^{14}C]methylamine influx were measured in the absence (□) or presence (■) of 5 μM L-glutamic acid. The purification of glutamate-binding proteins and their reconstitution into liposomes were performed as described under Materials and Methods. Ion flux assays were conducted, and the data obtained were analyzed according to the procedures described in Figure 1A. All values shown are the mean (±SEM) from five separate protein purification and reconstitution experiments. The constants estimated by fitting the data for glutamate-induced methylamine flux to a pseudo-first-order equation are k = 8.6 s^{-1} and I_∞ = 1173 nmol/mg of protein. Values of glutamate-induced flux that are significantly different from background flux are indicated (one asterisk, $p \leq 0.05$; two asterisks, $p \leq 0.01$, 8 df). (B) Determination of concentration-dependent stimulation by L-glutamate of [^{14}C]methylamine influx was performed as described in Figure 1B. The values shown are the mean net influx at 100 ms from three to four protein isolation and and reconstitution experiments. The constants estimated from the optimal fit to the Michaelis–Menten type of equation are K_{act} = 0.32 μM and I_{max} = 547 nmol/mg of protein.

one estimated for glutamate activation of ion flux in liposomes reconstituted with synaptic membrane proteins (K_{act} = 0.2 μM). However, the estimated net maximal transport activity at 100 ms in liposomes reconstituted with partially purified glutamate-binding proteins was approximately 5 times that determined for liposomes with synaptic membrane proteins.

Glutamate Receptor Agonist and Antagonist Effects on [^{14}C]Methylamine Flux into Proteoliposomes with Glutamate-Binding Proteins. Influx of [^{14}C]methylamine into proteoliposomes reconstituted with the glutamate-binding proteins was activated by NMDA as well as L-glutamate (Figure 3). The rate coefficient for NMDA-induced methylamine flux was approximately one-fifth that for the glutamate-activated cation flux in these liposomes (Figure 3). Quisqualate at 10 μM final concentration produced only a small, transient increase above background during the first 100 ms. This increase in methylamine flux above background was not maintained in the 200–800-ms period (data not shown). Kainic acid did not increase methylamine flux above background during any period of reaction (data not shown). These results were indicative of activation by L-glutamate of a receptor–ion channel complex in these liposomes that may

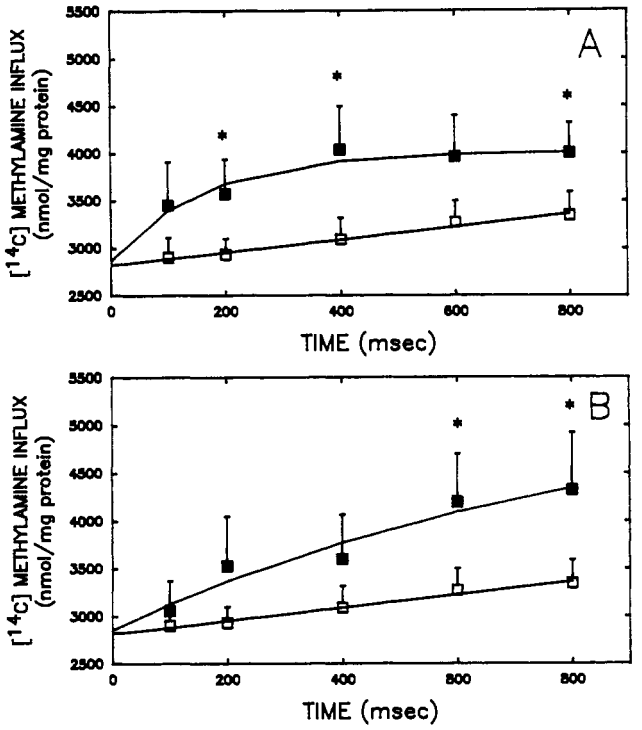

FIGURE 3: L-Glutamate and NMDA activation of [^{14}C]methylamine influx into liposomes reconstituted with partially purified glutamate-binding proteins. Rapid kinetics of methylamine flux were measured in the presence (■) or absence (□) of 10 μM L-glutamate (A) or in the presence (■) or absence (□) of 10 μM NMDA (B). Each value represents the mean (±SEM) from six protein isolation and reconstitution experiments. Both glutamate- and NMDA-stimulated flux as well as background [^{14}C]methylamine flux were measured in the same preparations. Values of glutamate- and NMDA-induced flux that are significantly different from background flux are indicated (asterisk, $p \leq 0.05$, 10 df). Data analysis and curve-fitting were performed as described in Figure 1A. Estimated constants for glutamate-activated ion flux are k = 6.1 s^{-1} and I_∞ = 1152 nmol/mg of protein; those for NMDA-stimulated methylamine flux are k = 1.2 s^{-1} and I_∞ = 2412 nmol/mg of protein.

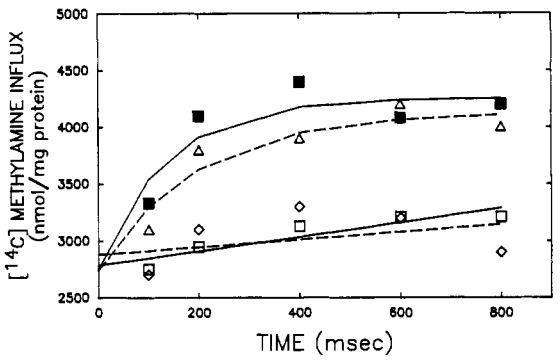

FIGURE 4: Effect on L-glutamate-stimulated [^{14}C]methylamine flux of the receptor antagonists 2-amino-5-phosphonopentanoic acid and L-glutamate diethyl ester. Rapid kinetics assays and data analysis were performed as described in Figure 3. The incubations were conducted in the presence of either 10 μM L-glutamate (■), or 10 μM L-glutamate plus 2 mM glutamate diethyl ester (△), or 10 μM L-glutamate plus 0.5 mM 2-amino-5-phosphonopentanoic acid (◇) and in the absence of all agents (□). The receptor antagonists had no effect on background [^{14}C]methylamine flux. The estimated constants for glutamate-activated methylamine flux are k = 7.4 s^{-1} and I_∞ = 1502 nmol/mg of protein, those for glutamate-activated flux in the presence of 2 mM L-glutamate diethyl ester are k = 5.3 s^{-1} and I_∞ = 1391 nmol/mg of protein. The values of [^{14}C]-methylamine flux shown are the mean from three to five protein isolation and reconstitution experiments.

be related to the NMDA rceptor, although the presence of a quisqualate-sensitive ion channel that is rapidly inactivated by the continuous presence of the agonist cannot be excluded.

Further evidence for the presence of an NMDA receptor–ion channel complex was sought by examining the effects of selective receptor antagonists on L-glutamate-induced methylamine flux into proteoliposomes. The quisqualate receptor antagonist L-glutamate diethyl ester did not alter substantially either the rate of glutamate-activated methylamine flux or the I_∞ produced by 10 μM L-glutamate (Figure 4). On the other hand, the NMDA inhibitor 2-amino-5-phosphonopentanoic acid completely inhibited the flux activated by L-glutamate (Figure 4). Very high concentrations of these two agents were used in these assays, ones that corresponded to the concentrations present in the quench solution used in all rapid kinetic determinations.

L-Glutamate-Induced S^{14}CN Uptake into Proteoliposomes Reconstituted with Glutamate-Binding Proteins. In all ion flux assays described above, we employed salts that contained mostly membrane-impermeable or slowly permeable anions, such as sulfate. Thus, permeation of cations such as methylamine or Na^+ through the proteoliposome membrane would probably not be accompanied by kinetically equivalent permeation of counterions. If a highly lipid-permeable anion such as SCN^- is employed, its distribution across the liposome membrane would be determined by its chemical gradient as well as by the movement of cations. Under such conditions, SCN^- would function as a probe for changes in cation permeation through the liposome bilayer and would provide another measure of L-glutamate-activated cation flux.

The flux of S$^{14}CN^-$ was measured under both basal and L-glutamate-induced Na^+ flux conditions in proteoliposomes reconstituted with partially purified glutamate-binding proteins (Figure 5A). The kinetics of S$^{14}CN^-$ flux were determined primarily for proteoliposomes incubated in a medium that contained 12.5 mM Na_2SO_4, final concentration. Nearly identical results were obtained when 5 mM methylamine was used in place of Na_2SO_4 (data not shown). Background SCN^- flux was a linear process within the incubation period of 100–800 ms. Finally, the enhancement of S^{14}CN flux produced by L-glutamate was dependent on the concentration of glutamic acid. The estimated K_{act} for glutamate-induced SCN^- flux in proteoliposomess reconstituted with the binding proteins was 0.47 μM (data not shown).

Activation of S$^{14}CN^-$ flux was induced not only by L-glutamate but also by the NMDA receptor agonists NMDA and ibotenic acid (Figure 5B,C). Ibotenic acid is a glutamate analogue with partial selectivity for the NMDA receptor (Watkins & Evans, 1981). Quisqualic acid at the same concentration as ibotenic acid and NMDA produced a very small and short-lived activation of S$^{14}CN^-$ in the same proteoliposome preparations (Figure 5D). As was observed with NMDA-enhanced methylamine flux, the rate coefficient for NMDA-stimulated SCN^- flux was approximately one-fifth that of the coefficient estimated for glutamate-stimulated SCN^- flux (Figure 5). On the other hand, the rate coefficient for ibotenate-induced SCN^- flux was 2 times that for L-glutamate-stimulated flux, but the net SCN^- flux activated by ibotenic acid was considerably smaller (Figure 5). The rate coefficients for both NMDA- and L-glutamate-induced SCN^- flux were approximately half of those estimated for [^{14}C]-methylamine flux into liposomes reconstituted with glutamate-binding proteins. The slower kinetics of SCN^- flux are probably due to the fact that this anion flux is a secondary process brought about by the initial influx of cations.

Analysis of the Proteins in the Glutamate-Binding Protein Liposomes and of the Role of the 71- and 63-kDa Proteins

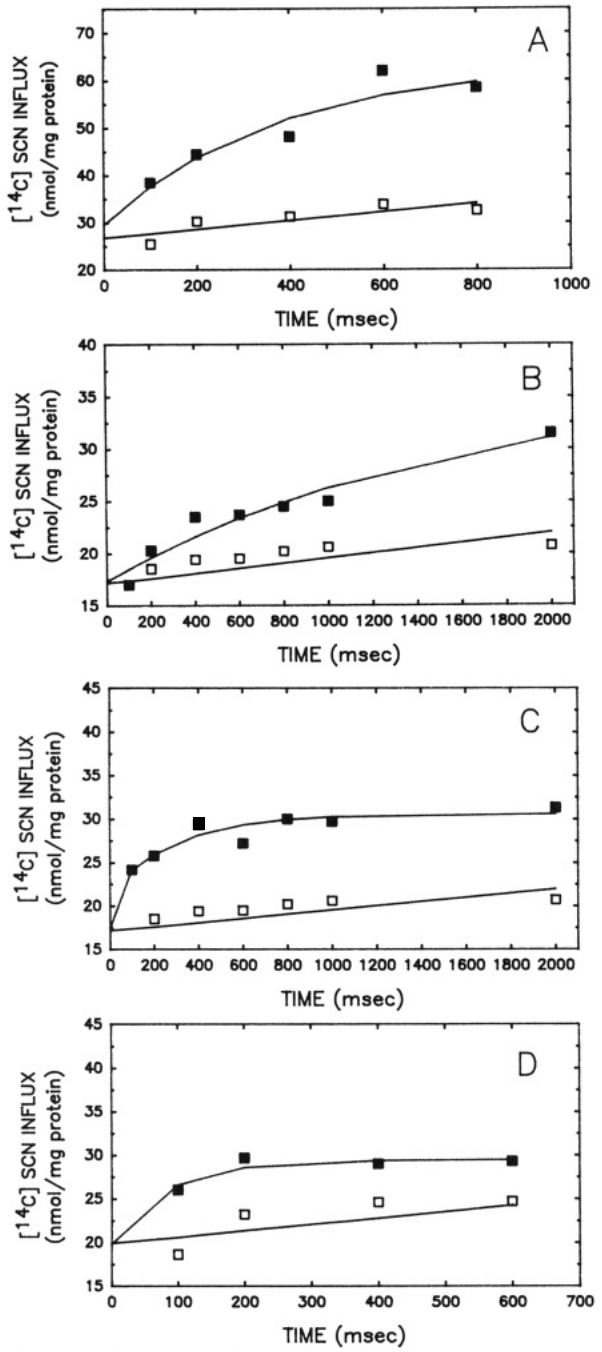

FIGURE 5: Characterization of the rapid kinetics of activation by receptor agonists of $S^{14}CN$ influx into liposomes reconstituted with partially purified glutamate-binding proteins. Rapid kinetic measurements were obtained under conditions identical with those described in Figure 2A except for the inclusion of Na_2SO_4 and KSCN into the incubation medium as described under Materials and Methods. All data shown are mean values of SCN^- flux from four to six protein purification and reconstitution experiments. All data were analyzed by fitting to pseudo-first-order rate equations as described in Figure 1A. The estimated rate coefficients are 2.7 s^{-1} for 5 μM L-glutamate (■ in A), 0.63 s^{-1} for 10 μM NMDA (■ in B), 6.5 s^{-1} for 10 μM ibotenate (■ in C), and 12.1 s^{-1} for 10 μM quisqualate acid (■ in D). Flux of $S^{14}CN$ in the absence of any agonists (□) is also shown.

in Glutamate-Initiated Ion Flux. Examination by SDS–PAGE and silver staining of the proteins present in liposomes reconstituted with the partially purified glutamate-binding protein fractions were indicative of a high degree of enrichment of proteins with a molecular size of approximately 67–70 kDa (Figure 6A, lanes 2 and 3). In some experiments, these proteins migrated in SDS gels at slightly different positions depending on the amount of lipid associated with the proteins.

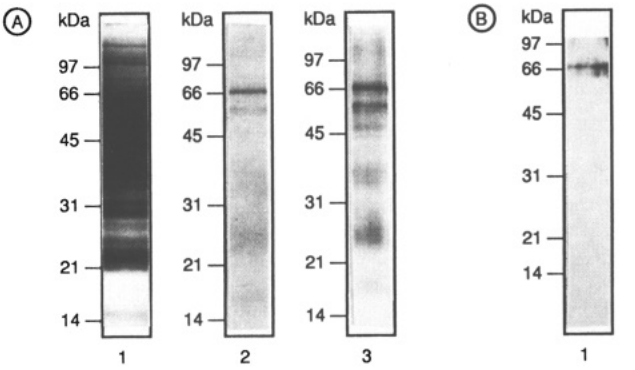

FIGURE 6: Protein composition of the glutamate-binding protein fraction determined by SDS–PAGE followed by silver staining and by immunostaining in Western blots. (A) Silver-stained electrophoretic pattern of synaptic membrane proteins (lane 1) and of the partially purified and liposome-reconstituted glutamate-binding protein fraction (lanes 2 and 3). The amount of protein loaded on each lane was 2.5 μg. The liposome-reconstituted protein fractions were dialyzed against 0.1% SDS in H_2O and lyophilized prior to dissolution in electrophoresis sample buffer. The migration of molecular weight marker proteins for each electrophoretic separation is indicated on the figure. The partially purified preparations of protein used for electrophoresis are from two different reconstitution experiments and are typical of most such experiments. (B) Electrotransfer of glutamate-binding proteins and immunostaining with antibodies raised against the 71- and 63-kDa glutamate-binding proteins. An aliquot (2.5 μg) of glutamate-binding proteins was subjected to electrophoresis and electrotransfer to nitrocellulose filter. Reaction with the antiserum and staining with the alkaline phosphatase substrate were performed as described under Materials and Methods.

Although in some experiments a 67–70-kDa and a 59–62-kDa band were the predominant proteins detected, in most preparations, there were several other prominent bands detected by SDS–PAGE analysis. The proteins most frequently observed had estimated molecular sizes of 59–62, 51–54, 40–42, 33–36, and 24–25 kDa (Figure 6A). The silver-stained bands for some of these proteins tended to be diffuse and may have included several proteins or may have represented microheterogeneity of each protein species.

To ascertain the relationship of the 67–70-kDa proteins in reconstituted liposomes to the glutamate-binding proteins that we had previously purified and characterized (Chen et al., 1988), we used antibodies raised against the 71- and 63-kDa glutamate-binding proteins (Eaton et al., 1990) in Western blots of the isolated proteins (Figure 6B). These antibodies have been shown to have high selectivity in recognizing the glutamate-binding proteins in immunoassays and to label primarily a protein band with a molecular size equal to 66–70 kDa in both synaptic membranes and purified preparations of the glutamate-binding proteins (Eaton et al., 1990). Furthermore, these antibodies were previously used to purify the 71- and 63-kDa proteins by immunoaffinity chromatographic procedures and to immunoextract 55–65% of the glutamate-binding entities from solubilized synaptic membrane protein preparations (Eaton et al., 1990). As is shown in Figure 6B, the antibodies reacted strongly with a 67–70-kDa protein band in the liposome preparations reconstituted with the partially purified glutamate-binding proteins.

Since most of the preparations of partially purified glutamate-binding proteins used in these reconstitution experiments contained proteins other than the 67–70-kDa proteins, we probed the contribution of the 67–70-kDa glutamate-binding proteins to the glutamate-activated [^{14}C]methylamine flux by prereacting the liposomes with the antibodies against these proteins. In these studies, the purified IgG fraction from preimmune and immune sera was used, and the liposomes were

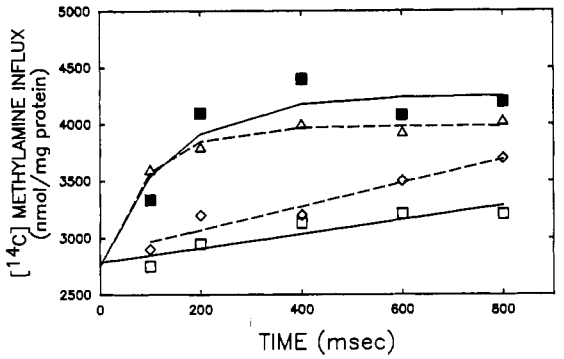

FIGURE 7: Effects of preincubation with immune and preimmune IgG's on L-glutamate-induced [^{14}C]methylamine flux in liposomes reconstituted with partially purified glutamate-binding proteins. Proteoliposomes were preincubated for 1 h at 4 °C with either buffer (■) or 5 μg/mL preimmune IgG (△) or 5 μg/mL immune IgG (◇). These proteoliposomes were subsequently employed in determinations of [^{14}C]methylamine flux in the presence or absence of 10 μM L-glutamic acid. Since preincubation with either type of IgG had no effect on background flux, the data were combined to obtain a common background flux of methylamine. Each value shown is the mean from four protein isolation and reconstitution experiments. The constants for glutamate-activated flux are $k = 7.4\ s^{-1}$ and $I_\infty = 1502$ nmol/mg of protein; those for glutamate-activated flux in the presence of preimmune IgG are $k = 10.9\ s1^{-1}$ and $I_\infty = 1232$ nmol/mg of protein. Methylamine flux in samples incubated with L-glutamate following preincubation with immune IgG was a linear process.

preincubated with the IgG fraction (5 μg/mL) for 1 h at 4 °C. We have previously shown that under these conditions of incubation the immune IgG inhibits greater than 80% of the glutamate-binding activity of isolated binding proteins (Eaton et al., 1990). Preincubation of liposomes with immune IgG produced a marked decrease in the rate of glutamate-activated [^{14}C]methylamine flux (Figure 7). Treatment with immune or preimmune IgG had no effect on background [^{14}C]methylamine flux. The background methylamine flux shown in Figure 7 is the average of the buffer, immune IgG, and preimmune IgG treatment conditions. Incubation of liposomes with preimmune IgG produced very small effects on glutamate-induced [^{14}C]methylamine flux (Figure 7).

Discussion

Several experimental approaches were developed in the overall effort to solubilize, partially purify, and reconstitute functional glutamate-activated cation channels from brain synaptic membranes. We can conclude from the studies described in this paper that the nonionic detergents Triton X-100 and *n*-octyl glucoside can be used successfully in such procedures. Many experiments were performed under a variety of solubilization/reconstitution conditions with the ionic detergent sodium cholate, but functional reconstitution of glutamate-activated Na^+ channels was never achieved (unpublished observations). This is in contradistinction to the apparently successful solubilization and reconstitution of brain synaptic membrane L-glutamate transport carriers using sodium cholate and asolectin and lack of successful reconstitution when Triton X-100 was employed during solubilization of the transport carriers (Kanner & Sharon, 1978b).

Crucial to successful reconstitutions of glutamate-sensitive ion channels solublized by either Triton X-100 or *n*-octyl glucoside is the presence in the solubilization buffer of either a neutral phospholipid, such as phosphatidylcholine or PE, at a concentration of 10–20 mg/mL or a low concentration of neutral phospholipids (0.1–0.5 mg/mL) plus 10% glycerol. The solubilization medium that contains low concentrations of PE (0.5 mg/mL) plus 10% glycerol can be used easily with chromatographic procedures designed for the purification and subsequent functional reconstitution of glutamate-binding proteins, therefore, this solubilization medium was used in all experiments described in this paper.

The use of [^{14}C]methylamine as the permeant ion through putative glutamte-activated ion channels and the employment of quench-flow, rapid kinetic measurements of ion fluxes represent methodological advances in the detection of functionally reconstituted glutamate receptor–ion channels. By measuring the increase in permeability for [^{14}C]methylamine, we were able to obtain estimates of glutamate-activated ion fluxes in liposomes reconstituted with synaptic membrane proteins without any apparent contribution to these fluxes by glutamate transport carriers. The kinetics of glutamate activation of ion channels could be described as a pseudo-first-order rate process. Maximum flux (I_∞) was achieved within 200–400 ms, whereas in the absence of glutamate [^{14}C]methylamine flux was a slow, linear process.

In liposomes reconstituted with synaptic membrane proteins, the estimated rate coefficients for ion fluxes due to glutamate activation of ion channels were not descriptive of a single process. The estimated rates for ion flux are probably the result of rates of binding of glutamate with various receptor subtypes present in neuronal membranes, an average of rates of ion permeation through different types of ion channels associated with these receptors, and the end product both of the rates of activation and of densensitization of different types of glutamate receptors. Reports from different laboratories have identified different rates of desensitization for various glutamate receptors, with the AMPA or quisqualate-sensitive ones having the most rapid rates of desensitization, on the order of 5–100 ms (Kiskin et al., 1986; Franke et al., 1987; Trussell et al., 1988). Since both kainate and AMPA/quisqualate receptors are apparently formed by homopolymers of isoforms of the recently cloned 100-kDa kainate receptor protein (Keinanen et al., 1990; Boulter et al., 1990), the differential desensitization rates are not an indication of different receptors for kainate and AMPA/quisqualate but of homopolymers formed by different isoforms of the 100-kDa protein (Sommer et al., 1990). Kainate-activated responses desensitize at a much slower rate than quisqualate-induced responses (Kiskin et al., 1986; Trussell et al., 1988; Sommer et al., 1990), therefore, it is likely that only desensitization of glutamate-activated, quisqualate-sensitive responses in liposomes reconstituted with membrane proteins may have contributed significantly to the estimated rates obtained by rapid kinetic measurements (100–800-ms period).

Methylamine fluxes produced by L-glutamate in liposomes reconstituted with partially purified glutamate-binding proteins had nearly identical rate coefficients with ion fluxes measured in liposomes reconstituted with synaptic membrane proteins. However, these two types of liposomes differed in the estimated maximal flux values activated by L-glutamate. The liposomes with glutamate-binding proteins had approximately 5–7 times higher maximum methylamine flux in response to glutamate as compared to liposomes reconstituted with synaptic membrane proteins. This enrichment in ion flux activity does not begin to approximate the nearly 200 times greater glutamate-binding activity associated with the partially purified preparation of glutamate-binding proteins when compared with synaptic membrane proteins (Chen et al., 1988). This discrepancy between enrichment in glutamate-binding and ion flux activity may be due to either protein denaturation during purification, inappropriate orientation of reconstituted glutamate-binding proteins, or formation of leaky liposomes upon

reconstitution of these proteins. The experimental evidence we have accumulated was indicative of substantial leakiness of the liposomes reconstituted with partially purified glutamate-binding proteins. Nevertheless, despite this increase in background cation flux, these liposomes were useful in probing the characteristics of glutamate-activated ion flux.

The fact that the rate coefficients for L-glutamate-induced [^{14}C]methylamine fluxes in liposomes reconstituted with partially purified glutamate-binding proteins were very similar to those determined in liposomes reconstituted with solubilized synaptic membrane proteins may be an indication that the purified preparations contain all of the subtypes of glutamate receptors found in synaptic membranes. Alternatively, it may be an indication that ion fluxes in both liposomes reconstituted with membrane proteins and those with partially purified glutamate-binding proteins are due to a predominant set of receptor–ion channel proteins. The latter possibility seems to be supported by the following observations. The rate coefficients for glutamate-induced methylamine flux and the estimated K_{act} for L-glutamate activation of fluxes were nearly identical in both sets of liposomes. Furthermore, the pharmacological characteristics of the glutamate-activated ion flux into liposomes reconstituted with the partially purified glutamate-binding protein fraction fit those of one subtype of receptor, the NMDA receptor.

As was described earlier, Sokolovsky and his colleagues (Ikin et al., 1990) have isolated a complex of proteins apparently related to the NMDA receptor–ion channel. We have found that in most of our preparations there were proteins with estimated molecular sizes fairly similar to those described by Ikin et al. (1990) such as the 67–70, 59–62, 40–42, and 33–36-kDa proteins (Figure 6). The NMDA-induced ion fluxes and the sensitivity of such fluxes to inhibition by 2-amino-5-phosphonopentanoic acid may be the result of functional reconstitution of these proteins. The marked decrease in the initial rate of glutamate activation of ion fluxes by antibodies to the 71- and 63-kDa proteins may be the result of immunochemical homologies between the types of proteins that make up the NMDA receptor–ion channels, in particular the M_r 67000 protein identified by Ikin et al., (1990) and the 71- and 63-kDa glutamate-binding proteins. Alternatively, the 71-kDa glutamate-binding protein we had previously isolated may be the glutamate recognition protein of an NMDA receptor–ion channel complex in synaptic membranes.

In very recent studies, we have found that elution of glutamate-binding proteins from the affinity chromatography matrix by 3 mM NMDA produced high enrichment of the same complex of five proteins as those eluted by 5 mM glutamate, i.e., the 69-, 60-, 40-, 35-, and 25-kDa proteins. Furthermore, when these proteins were reconstituted in liposomes, both NMDA and L-glutamate (10 μM) produced activation of [^{14}C]methylamine influx that was identical with that described in this paper. Finally, the activation of flux produced by exposure of the liposomes to 10 μM NMDA was markedly inhibited by pretreatment of the liposomes with the anti-71-kDa protein antibodies (unpublished observations). Since the glutamate-activated cation flux was also completely inhibited by 2-amino-5-phosphonopentanoic acid, but not by glutamate diethyl ester, it appears that the ~70-kDa glutamate-binding protein is a component of the NMDA receptor complex.

If the ~70-kDa proteins are a component of the NMDA receptor complex, then we need to explain the very weak binding of NMDA to the purified 71- and 63-kDa proteins observed previously (Chen et al., 1988). One possibility is that separation of the 71-kDa protein from the other protein subunits that form the complex of the NMDA receptor–ion channel alters the ligand-binding properties of the protein. The binding of NMDA to the ~70-kDa glutamate-binding protein may be strongly dependent on the interaction of this protein with other subunits. Solubilization of synaptic membrane proteins in the presence of lipids and glycerol, i.e., the method used in the reconstitution studies, may preserve the multisubunit structure of glutamate/NMDA receptor–ion channel complexes and the sensitivity of the complexes to activation by NMDA.

The binding of quisqualic acid to the purified 71-kDa protein that we reported previously (Chen et al., 1988) may represent a low-affinity interaction of this ligand with the glutamate recognition site of the protein and may be unrelated to interactions of quisqualate with an AMPA-sensitive receptor–ion channel. This conclusion is based on the observation that the binding of quisqualate to the isolated 71-kDa protein has an estimated K_D that is at least 350 times higher than the K_{act} for quisqualate-induced opening of ion channels in neuronal membranes (Perouansky & Grantyn, 1989). Furthermore, AMPA, which is a more selective agonist for the AMPA/quisqualate receptors, did not bind to the isolated glutamate-binding proteins (Chen et al., 1988). Finally, we have never seen enrichment in our purified protein preparations of a ~100-kDa protein that may correspond to the kainate/AMPA receptor.

We consider the results presented in this paper as initial evidence for the successful partial purification and reconstitution of L-glutamate/NMDA receptor–ion channels. We are currently accumulating further evidence for reconstitution of glutamate-sensitive ion channels by the use of planar lipid bilayer membranes and direct electrical measurements of ion channel activity (Uto et al., 1990; unpublished observations). The further definition of the precise protein composition of such receptors will be achieved either by proceeding to higher levels of purification of the protein complexes and application of the reconstitution methodologies we have developed or by cloning the cDNAs for these proteins and expressing them in cells such as the *Xenopus* oocyte.

Acknowledgments

We thank M. Eaton for providing the antibodies used in these studies, N. Marioli for technical assistance with the reconstitution and ion flux studies, L. Kunkle for typing the manuscript, and Dr. M. L. Michaelis for reviewing this paper. We acknowledge the support provided by the Center for Biomedical Research, The University of Kansas.

Registry No. NMDA, 6384-92-5; methylamine, 74-89-5; L-glutamic acid, 56-86-0; sodium, 7440-23-5; thiocyanate, 302-04-5.

References

Anwyl, R. (1977) *J. Physiol. 273*, 367–388.
Bekkers, J. M., & Stevens, C. F. (1989) *Nature 341*, 230–233.
Blake, M. S., Johnston, K. H., Russell-Jones, G. T., & Gotschlich, E. C. (1984) *Anal. Biochem. 136*, 175–179.
Boulter, J., Hollmann, M., O'Shea-Greenfield, A., Hartley, M., Deneris, E., Maron, C., & Heinemann, S. (1990) *Science 240*, 1033–1037.
Chang, H. H., & Michaelis, E. K. (1980) *J. Biol. Chem. 255*, 2411–2417.
Chen, J.-W., Cunningham, M. D., Galton, N., & Michaelis, E. K. (1988) *J. Biol. Chem. 263*, 417–427.
Cotman, C. W., & Iversen, L. L. (1987) *Trends Neurosci. 10*, 263–302.

Cotman, C. W., Monaghan, D. T., & Ganong, A. H. (1988) *Annu. Rev. Neurosci. 11*, 61–80.
Cull-Candy, S. G., & Usowicz, M. M. (1987) *Nature 325*, 525–528.
Cunningham, M. D., & Michaelis, E. K. (1990) *J. Biol. Chem. 265*, 7768–7778.
Curtis, D. R., & Johnston, G. A. R. (1974) *Ergeb. Physiol. 69*, 97–188.
Eaton, M. J., Chen, J. W., Kumar, K. N., Cong, Y., & Michaelis, E. K. (1990) *J. Biol. Chem. 265*, 16195–16204.
Fagg, G. E., & Matus, A. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 6876–6880.
Foster, A. C., & Fagg, G. E. (1984) *Brain Res. Rev. 7*, 103–164.
Franke, C., Hatt, H., & Dudel, J. (1987) *Neurosci. Lett. 77*, 199–204.
Garty, H., Rudy, B., & Karlish, S. J. D. (1983) *J. Biol. Chem. 258*, 13094–13099.
Hollman, M., O'Shea-Greenfield, A., Rogers, S. W., & Heinemmann, S. (1989) *Nature 342*, 643–648.
Huganir, R. L., & Racker, E. (1980) *J. Supramol. Struct. 4*, 419–425.
Ikin, A. F., Kloog, Y., & Sokolovsky, M. (1990) *Biochemistry 29*, 2290–2295.
Jahr, C. E., & Stevens, C. F. (1987) *Nature 325*, 522–525.
Johnson, J. W., & Ascher, P. (1987) *Nature 325*, 529–531.
Kanner, B. I., & Sharon, I. (1978a) *Biochemistry 17*, 1207–1212.
Kanner, B. I., & Sharon, I. (1978b) *FEBS Lett. 94*, 245–248.
Keinanen, K., Wisden, W., Sommer, B., Werner, P., Herb, A., Verdoorn, T. A., Sakmann, B., & Seeburg, P. H. (1990) *Science 249*, 556–560.
Kiskin, N. I., Khristahl, O. A., & Tsendrenko, A. Y. (1986) *Neurosci. Lett. 63*, 225–330.
Krnjevic, K. (1974) *Physiol. Rev. 54*, 418–540.
MacDermott, A. B., Mayer, M. L., Westbrook, G. L., Smith, S. J., & Barker, J. L. (1986) *Nature 321*, 519–522.
Michaelis, E. K., Belieu, R. M., Grubbs, R. D., Michaelis, M. L., & Chang, H. H. (1982) *Neurochem. Res. 7*, 417–430.
Michaelson, D., & Raftery, M. A. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 4768–4772.
Monaghan, D. T., Yao, D., & Cotman, C. W. (1985) *Brain Res. 340*, 378–383.
Neuhoff, V., Phillipp, K., Zimmer, H., & Mesecke, S. (1979) *Hoppe-Seyler's Z. Physiol. Chem. 360*, 1657–1670.
Nowak, L., Bregestovski, P., Ascher, P., Herbert, A., & Prochiantz, A. (1984) *Nature 307*, 462–465.
Papahadjopoulos, D., Cowden, M., & Kimelberg, H. (1973) *Biochim. Biophys. Acta 330*, 8–26.
Perouansky, M., & Grantyn, R. (1989) *J. Neurosci. 9*, 70–80.
Peterson, G. L. (1977) *Anal. Biochem. 83*, 346–356.
Recasens, M., Guiramand, J., Nourigat, A., Sasetti, I., & Devilliers, G. (1988) *Neurrochem. Int. 13*, 463–467.
Sladeczek, F., Recasens, M., & Bockaert, J. (1988) *Trends Neurosci. 11*, 545–549.
Sommer, B., Keinanen, K., Verdoorn, T. A., Wisden, W., Burnashev, N., Herb, A., Kohler, M., Takagi, T., Sakmann, B., & Seeburg, P. H. (1990) *Science 249*, 1580–1585.
Sonders, M. S., Barmettler, P., Lee, J., Kitahara, Y., Keana, J. F. W., & Weber, E. (1990) *J. Biol. Chem. 265*, 6776–6781.
Suarez-Isla, B. A., Wan, K., Lindstrom, J., & Montal, M. (1983) *Biochemistry 22*, 2319–2323.
Sugiyama, H., Ito, I., & Watanabe, M. (1989) *Neuron 3*, 129–132.
Trussell, L. O., Thio, L. L., Zorumski, C. F., & Fishback, G. D. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 4562–4566.
Uto, M., Michaelis, E. K., Hu, I. F., Umezawa, Y., & Kuwana, T. (1990) *Anal. Sci. 6*, 221–225.
Vyklicky, L., Krusek, J., & Edwards, C. (1988) *Neurosci. Lett. 89*, 313–318.
Watkins, J. C., & Evans, R. H. (1981) *Rev. Pharmac. Toxicol. 21*, 165–204.
Watkins, J. C., Krogsgaard-Larsen, P., & Honore, T. (1990) *Trends Pharmacol. Sci. 11*, 25–33.
Wray, W., Boulikas, T., Wray, V. P., & Hancock, R. (1981) *Anal. Biochem. 118*, 197–203.