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Rapid Communication

# Functional Reconstitution of α-Amino-3-Hydroxy-5-Methylisoxazole-4-Propionate (AMPA) Receptors from Rat Brain

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**Abstract:** Glutamate receptors belonging to the subclass specifically activated by α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) were solubilized from rat forebrain membranes with Triton X-100 and partially purified through a series of three chromatographic steps. Specific [$^3$H]AMPA binding increased 30–60-fold during the isolation procedure. A protein band recognized by antibodies against specific amino acid sequences of the glutamate receptor-A subunit was enriched with each purification step; the molecular mass of this band (105 kDa) corresponded to that of cloned AMPA receptor subunits. Photoaffinity labeling of forebrain membranes with 6-cyano-7-[$^3$H]nitroquinoxaline-2,3-dione, a specific antagonist of the AMPA receptor, labeled a single band that comigrated with the immunolabeled protein. On reconstitution of the partially purified material into bilayer patches, single-channel current fluctuations were elicited by 300 n*M* AMPA and blocked by 1 μ*M* 6,7-dinitroquinoxaline-2,3-dione. **Key Words:** α-Amino-3-hydroxy-5-methylisoxazole-4-propionic acid-type glutamate receptor—Ligand-gated ion channel—Affinity labeling—6-Cyano-7-nitroquinoxaline-2,3-dione—Single-channel activity—Artificial bilayer patch. **Bahr B. A. et al.** Functional reconstitution of α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) receptors from rat brain. *J. Neurochem.* **59,** 1979–1982 (1992).

Recent studies indicate that glutamate receptors (GluRs) of the α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) subclass can exist in different functional states in telencephalic synapses. Solubilization of membrane fractions prepared from forebrain of adult rats results in a marked decrease in low-affinity binding and an increase in the number of high-affinity sites (Hall et al., 1992; Hunter and Wenthold, 1992). Quantitative analyses incorporating corrections for lost receptors suggest that these effects reflect a simple conversion of the AMPA receptor from one affinity state to another (Hall et al., 1992). Therefore, it appears that unknown factors in the synaptic complex maintain the AMPA receptor in a low-affinity state. There is also evidence that the decay time constant of AMPA receptor-mediated synaptic responses changes in association with long-term potentiation in the hippocampus (Ambros-Ingerson et al., 1991). Because the decay time constant relates to the mean open time of receptors (Magelby and Stevens, 1972), these results imply that the functional properties of the AMPA receptor/ionophore complex are regulated by elements in the synaptic environment that are sensitive to long-term potentiation-inducing stimulation. Given that AMPA receptors are likely to mediate fast excitatory responses at many sites in forebrain, it is important to characterize these modulatory agents.

One approach to this problem would be to analyze the effects of candidate mechanisms on purified receptors reconstituted into artificial membranes. Shimazaki et al. (1992) recently purified an AMPA binding protein from bovine cerebellum using spider toxin affinity chromatography and showed that it has glutamate-induced channel activity after reconstitution. The relationship of this protein to synaptic AMPA receptors found in telencephalon is unclear in the absence of further characterization. An AMPA-type receptor from *Xenopus* brain has also been purified and reconstituted but is reported to have a molecular mass much different than that for AMPA receptor subunits found in rat brain (Ambrosini et al., 1991; Henley et al., 1992). Here we describe a simple procedure based on purification schemes described by Hunter and Wenthold (1992) that results in a 30–60-fold purification of rat forebrain

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*Abbreviations used:* AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; DEAE, diethylaminoethyl; DNQX, 6,7-dinitroquinoxaline-2,3-dione; GluR, glutamate receptor; PEI, polyethyleneimine; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; WGA, wheat-germ lectin affinity.

PA receptors and that yields functional receptors after nstitution.

## MATERIALS AND METHODS

### ification of the rat brain AMPA receptor

rains from adult rats (Sprague–Dawley) were rapidly re-ved following anesthesia and decapitation, and forebrain nbranes were prepared as previously described (Hall et 1992). The membranes were solubilized in ice-cold er A (30 m*M* HEPES, 5 m*M* EDTA, 1 m*M* EGTA, and % $NaN_3$, pH 7.4) with 1% (wt/vol) Triton X-100, 20% vol) glycerol, and 80 m*M* KSCN, at a detergent:protein o of 3.2. The mixture was incubated on ice for 30 min, ted twofold with buffer A, and incubated at 37°C for ther 30-min period with constant agitation. The particu- matter was subsequently removed by centrifugation at 00 *g* for 3 h. The supernatant was diluted with an equal me of buffer A with 10% glycerol and applied to a diethninoethyl (DEAE)-Sepharose column (5 × 15 cm) equili-ed at 4°C with buffer A containing 1% *n*-octylglucoside ehringer-Mannheim), 10% glycerol, and 0.05% phostidylcholine (buffer B). The column was washed at a rate of 6 ml/min with 4 column volumes of buffer B eluted with a 700-ml linear salt gradient from 0 to 250 f KSCN.

ollected fractions were tested for AMPA binding in a dard assay in which samples are incubated for 1 h at with 50 n*M* [$^3$H]AMPA (60 Ci/mmol; NEN/Du Pont) he presence of 50 m*M* KSCN and then filtered through s fiber filters (Bahr et al., 1992). Protein content was sured according to the technique of Bradford (1976). ctions containing AMPA receptor were pooled and ap-d to a 1.5- × 11-cm wheat-germ lectin affinity (WGA) mn at 4°C. After the column was washed with 200 ml uffer B containing 0.1 *M* NaCl, the AMPA receptor was ed with 0.7 *M N*-acetyl-D-glucosamine in buffer B. Frac-s containing [$^3$H]AMPA binding activity were pooled GA pool), and a portion was injected into an HPLC poly-lenimine (PEI) anion-exchange column (1 × 12.5 cm; herey-Nagel) equilibrated at 23°C in buffer B. After the mn was washed, a linear gradient from 0 to 250 m*M* CN was used to elute the AMPA receptor (PEI pool).

### ctrophoresis, immunoblot, and photoaffinity eling

amples from each purification step were concentrated in tricon devices (Amicon) and subjected to sodium do-yl sulfate–polyacrylamide gel electrophoresis (SDS-GE), and separated proteins were either silver-stained or sferred to nitrocellulose (pore size, 0.2 μm) for 8–16 h rnette, 1981). Nitrocellulose sheets were immuno-ned with antibodies (anti-GluR) against two peptides responding to amino acids 163–181 and 167–188 of the luR-1" receptor (Hollmann et al., 1989) as previously cribed (Bahr et al., 1992). Antibodies were obtained m rabbits that had been injected first with peptides con-ated to keyhole limpet protein and then with free pep-s; the antibodies were affinity-purified with immobi-d peptides.

ysed $P_2$ membranes (2 mg of protein/ml) were photoaf-ty-labeled by irradiating them at 254 nm for 2 h at 0°C he presence of 100 n*M* 6-cyano-7-[$^3$H]nitroquinoxaline--dione ([$^3$H]CNQX; 17 Ci/mmol; NEN/Du Pont). The mbranes were subsequently washed by centrifugation and processed for immunoblotting using anti-GluR antibodies.

### Receptor reconstitution into lipid bilayers

Partially purified AMPA receptors were treated with 0.1 mg/ml of phosphatidylserine and 2 mg/ml of phosphatidylcholine (Avanti Polar Lipids) and subsequently diluted with an equal volume of buffer A with 10% glycerol to reduce the *n*-octylglucoside concentration to 0.5%. The receptor pool had a final [$^3$H]AMPA binding activity of 2–6 pmol/ml and was stored at −80°C after quick-freezing in liquid nitrogen. Receptors were reconstituted either into large (1-mm$^2$) solvent-free or small "tip-dip" bilayers as previously reported (Vodyanoy et al., 1987; Vodyanoy, 1989, 1991). The lipid 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine (Avanti) was typically used to form a bilayer on the aperture of a bilayer chamber or on the tip of a patch pipette; receptors (20 μl) were then added to the bulk solution (3 ml) bathing the *cis* side of the preformed bilayer. The small patch bilayers were formed in "outside-out" asymmetric saline conditions with 110 m*M* KCl, 4 m*M* NaCl, 2 m*M* $NaHCO_3$, 0.1 m*M* $CaCl_2$, 1 m*M* $MgCl_2$, 2 m*M* MOPS inside and 125 m*M* NaCl, 5 m*M* KCl, 1.25 m*M* $NaH_2PO_4$, and 5 m*M* Tris (pH 7.4) outside. Finally, different concentrations of AMPA (Tocris Neuramin) were delivered to the *cis* side of the bilayer in the absence or presence of 6,7-dinitroquinoxaline-2,3-dione (DNQX; Cambridge Research Biochemicals). The single-channel events were recorded with a VCR system and subjected to computer analysis. Recorded signals were filtered at 1–5 kHz and sampled at 0.2–10-ms intervals.

## RESULTS AND DISCUSSION

More than 50% of the [$^3$H]AMPA binding sites in forebrain membranes were solubilized with 1% Triton X-100. After three purification steps, i.e., DEAE anion-exchange, WGA, and PEI anion-exchange chromatographies, specific [$^3$H]AMPA binding was increased 30–60-fold (Table 1). We previously used gel filtration to show that the partially purified receptor has a native molecular mass of ~425,000 in the presence of Triton X-100 (Hall et al., 1991) as reported earlier by Hunter et al. (1990). Silver-stained electrophoretic gels with the purified material revealed about six proteins between 80 and 140 kDa, the darkest stained of which was a 105–115-kDa band (Fig. 1C). Recovery of [$^3$H]AMPA binding from the PEI step was quite low, suggesting that

**TABLE 1.** *Partial purification of AMPA receptors from rat forebrain*

| Fraction | Total binding (pmol) | Total protein (mg) | Specific activity (pmol/mg) | Purification (-fold) | Yield (%) |
|---|---|---|---|---|---|
| Soluble | 450 | 273 | 1.7 ± 0.3 | 1 | 100 |
| DEAE | 213 | 8.1 | 26 ± 3 | 16 ± 4 | 48 ± 3 |
| WGA | 160 | 3.0 | 54 ± 5 | 33 ± 7 | 36 ± 4 |
| PEI | 13 | 0.2 | 65 ± 6 | 40 ± 9 | 3 ± 1 |

Data are mean ± SEM values compiled from four two-step (DEAE and WGA) and three three-step AMPA receptor preparations. Solubilized forebrain membranes (soluble fraction) were subjected to (a) DEAE anion-exchange, (b) WGA, and (c) PEI anion-exchange chromatographies as described in the text. Portions of the receptor pools were tested for [$^3$H]AMPA binding and protein content.

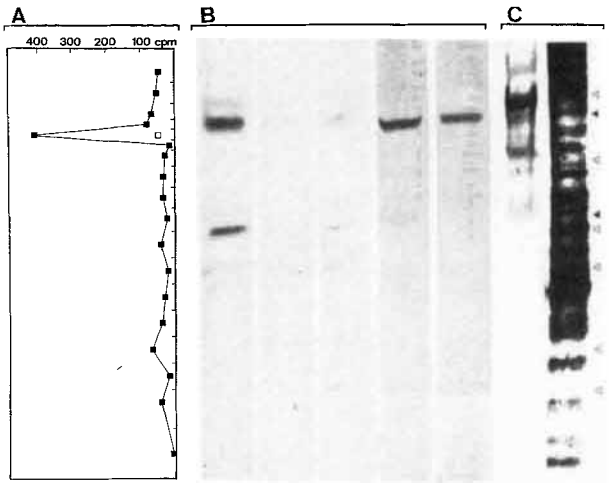


**FIG. 1.** Photoaffinity labeling, immunoblotting, and silver staining of the rat brain AMPA receptor. **A**: Photoaffinity labeling. Membranes were irradiated at 254 nm in the presence of [$^3$H]CNQX, washed, and subjected to immunoblotting. The nitrocellulose lane was subsequently analyzed for $^3$H by sectioning as indicated by ticks on the vertical axis and placing the strips in Liquiscint scintillation medium. Background of 40 cpm was subtracted from the averaged values from three lanes plotted in the figure. Additional samples containing 2 m*M* L-glutamate during irradiation were processed in parallel, and the $^3$H value found at the position of the 105-kDa immunostained band is shown (□). **B:** Protein samples were analyzed by immunoblot using anti-GluR versus (from left to right) 40 and 4 μg of forebrain membranes and 4, 4, and 3 μg of receptor pools from DEAE, WGA, and PEI chromatographic steps, respectively. **C:** Ten micrograms of solubilized membrane proteins (right lane) and 3 μg of purified material from the PEI chromatography step (left lane) were subjected to SDS-PAGE and silver staining. Open arrowheads show the positions of 116-, 84-, 58-, 48.5-, 36.5-, and 26.6-kDa standards, whereas solid arrowheads indicate the 105- and 59-kDa immunoreactive bands.

some binding activity was lost; however, many proteins of <80 kDa evident after WGA chromatography (data not shown) were apparently removed from the AMPA receptor following the PEI step (Fig. 1C, left lane). Immunoblots showed that a 105-kDa protein was increasingly labeled by anti-GluR antibodies across sequential chromatographic procedures (Fig. 1B); the molecular size of this antigen closely resembles that expected for cloned AMPA receptor subunits shown to bind AMPA when expressed in cultured cells (Hollmann et al., 1989; Keinanen et al., 1990). A smaller protein of 57–61 kDa was also labeled by anti-GluR antibodies as previously reported (Bahr et al., 1992). This antigen and the AMPA receptor copurified through the DEAE step but were separated by subsequent chromatographic procedures (Fig. 1B). However, a diffuse silver-stained band of ~60 kDa was still seen in the purified material of some receptor preparations. Finally, a 120–140-kDa protein appears to be recognized by anti-GluR antibodies early in the purification procedure. This band may correspond to the AMPA binding protein purified by spider toxin affinity chromatography described elsewhere (Shimazaki et al., 1992).

To obtain further evidence that the 105-kDa immunolabeled band is a component of AMPA-type glutamate receptors, membranes were photoaffinity-labeled with [$^3$H]-CNQX. This AMPA receptor antagonist contains an aromatic group, which presumably is activated by UV radiation to form a covalent attachment to the receptor. Membranes irradiated with UV light reliably showed specific incorporation of [$^3$H]CNQX (34,000 cpm/mg of protein, or 1.2 pmol/mg) into a protein that comigrated with the anti-GluR-labeled band (Fig. 1A). [$^3$H]CNQX incorporation was completely blocked when photoaffinity labeling was done in the presence of a saturating concentration of L-glutamate (2 m*M*) or CNQX (5 μ*M*) (see Fig. 1A). Quisqualate and AMPA were similarly effective, with $EC_{50}$ values of 5 and 50 μ*M*, respectively, whereas high concentrations of *N*-methyl-D-aspartate receptor ligands (150 μ*M N*-methyl-D-aspartate or 5 m*M* D-serine) had only minor effects (data not shown).

Scatchard analysis in solubilized forebrain membranes best resolved [$^3$H]AMPA binding into a single population of high-affinity binding sites with a $K_D$ value (15–30 n*M*) that matched previous reports (Hall et al., 1992; Hunter and Wenthold, 1992). Binding of 11 [$^3$H]AMPA concentrations between 10 and 1,500 n*M* to receptors from the WGA purification step also resolved into a single population of binding sites ($r = 0.953$) with $K_D$ and $B_{max}$ values of 15 n*M* and 53 pmol/mg of protein, respectively. The Hill coefficient determined from these binding studies was similar to that before fractionation ($n_H = 0.8–1.0$), and the binding activities were blocked by CNQX.

After reconstitution into large bimolecular membranes (pure lipid), partially purified AMPA receptors from the WGA and PEI steps were found to be associated with ligand-gated conductance activity. The conductance was increasingly activated by step concentrations of AMPA and was blocked by DNQX (Fig. 2A). When AMPA receptors from the PEI step were reconstituted into bilayer patches, the addition of 300 n*M* AMPA to the pseudoextracellular solution elicited single-channel current fluctuations as illustrated in Fig. 2B. The most distinctive current fluctuations were ~60 pS in conductance with a mean open time of ~18 ms. This closely resembles the high-conductance channels found in patch-clamp studies of hippocampal neurons (Jahr and Stevens, 1987; Tang et al., 1989, 1991). Analyses with higher time resolution found that the channel records in addition appear to contain current fluctuations produced by a low-conductance channel (~10 pS) with a mean open time of ~1 ms (V. Vodyanoy et al., submitted). The channel activity induced by AMPA appeared to be completely inhibited by 1 μ*M* DNQX (Fig. 2C), as in the case of receptors reconstituted in large membranes.

Membrane fractions prepared from rat forebrain contain both high- and low-affinity AMPA binding sites, with the latter being far more numerous (Olsen et al., 1987; Terramani et al., 1988; Hall et al., 1992). Solubilization of the membranes yields binding sites with comparable affinities but reverses the relative concentrations of the two affinity states (see Hall et al., 1992). The affinities and Hill coefficients for AMPA binding obtained in the present study are very similar to those reported for the high-affinity sites in membrane and solubilized preparations; thus, the procedures used to purify AMPA receptors do not appear to disturb significantly the ligand binding characteristics of the receptors. There is also evidence that the purified receptors retain at least certain of their in situ binding and conductance properties after reconstitution. Application of low concentrations of AMPA to the reconstituted receptors produced an increase in membrane conductance that was blocked by appropriate concentrations of a receptor antago-

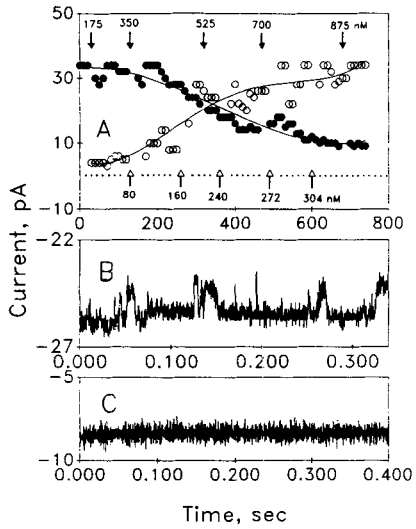


**FIG. 2.** Electrophysiological properties of reconstituted AMPA receptors. **A:** Membrane currents in large bilayers were measured as a function of AMPA (○) or DNQX (●) concentration. Partially purified material was added to preformed large bilayers 15 min before the start of the recording. AMPA (○) was added sequentially at the times marked by open arrows to give the total concentration (in n*M*) shown underneath each arrow; no AMPA was present before the first addition (voltage clamped at 6 mV). Alternatively, 600 n*M* AMPA was added before the start of the recording; DNQX (●) was then added sequentially to give the final concentrations shown above the closed arrows (voltage clamped at 20 mV). Zero current is indicated by a dotted line. Each recording has been repeated with comparable results in at least five separate reconstitutions using material from three different receptor preparations. **B and C:** Single-channel currents from receptors reconstituted in bilayer patches were elicited by addition of 300 n*M* AMPA in the absence (**B**) or presence (**C**) of 1 $\mu M$ DNQX. The voltage was clamped at −20.5 mV.

nist. Single-channel events were readily detected, and these had conductances and time courses not unlike those described for subgroups of AMPA receptors in cultured hippocampal neurons (Jahr and Stevens, 1987; Tang et al., 1989, 1991) and in cDNA functional expression systems using mammalian cells (Keinanen et al., 1990). Indeed, it appears that both the low- and high-conductance receptors (or receptor states) found in physiological experiments are present in the preparations described here. In all, it appears that purified/reconstituted receptors will be useful in testing hypotheses regarding the factors that regulate the functional properties of AMPA receptors.

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