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Author	鈴木, 岳之(Suzuki, Takeshi) 下遠野, 久美子(Shimotohno, Kumiko) Nakamura, Takeshi Brorson, James R.
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Quantitative expression of AMPA receptor subunits in HEK293 cells

Takeshi Suzuki[1]*, Kumiko Shimotohno[1], Takeshi Nakamura[2], James R Brorson[3]

[1]Department of Basic Biological Sciences, Kyoritsu University of Pharmacy,
[2]Department of Physiology, Juntendo University School of Medicine
[3]Department of Neurology, University of Chicago

Abstract

The participation of AMPA receptors in certain neurodegenerative diseases may depend on their Ca^{2+} permeability. AMPA receptors, which consist of multimetric assemblies of four (or five) subunits, can be divided into two subclasses, those with low (type I) and high Ca^{2+} permeability (type II). We transfected cDNAs of GluR1 and GluR2 AMPA receptor subunits in varying ratios into HEK293 cells to clarify the subunit ratio-dependence of glutamate responses. An implicit assumption in this study is that levels of protein subunit expression reliably reflect cDNA transfection ratios. Analysis of whole-cell currents generated from co-expression of GluR1 and GluR2 indicated that two functionally distinct receptors are produced (types I and II). When GluR1 and GluR2 were co-transfected in a 4:1 ratio, the resulting whole-cell current traces exhibited properties that were a mixture of those of type I and type II receptors. The experimental system of quantitative subunit protein expression developed in this study is useful for electrophysiological study of AMPA receptor functions.

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*Corresponding author: T Suzuki
E-mail: suzuki-tk@kyoritsu-ph.ac.jp
Address: 1-5-30 Shiba-koen, Minato-ku, Tokyo 105-8512, Japan
Phone: +81-3-5400-2496

The properties of α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors have important implications for synaptic physiology and for the pathophysiological roles played by these ligand-gated ion channels. The participation of AMPA receptors in certain neurodegenerative diseases may depend on their Ca^{2+} permeability. AMPA receptors, which consist of multimetric assemblies of four subunits, can be divided into two subclasses, those with low (type I AMPA receptor) and high Ca^{2+} permeability (type II). Early studies of cloned AMPA receptor subunits revealed the key role played by the GluR2 subunit, and by a single arginine residue at the 'Q/R site', in determining channel Ca^{2+} permeability (4,5,11). In native neurons expressing mixtures of Ca^{2+}-impermeable and Ca^{2+}-permeable AMPA subunits, some studies have presented evidence apparently consistent with stochastic subunit assembly, and with a strictly dominant role of GluR2 in suppression of divalent cation permeability (1,3,12).

Most evidence has favored a dominant role for GluR2 in determining the properties of AMPA receptors, with a single copy of GluR2 sufficient to produce receptor assemblies with low Ca^{2+} permeability (2,12). Furthermore, AMPA receptor subunits expressed in heterologous systems have appeared to be indiscriminate in their co-assembly, with each of the subunits capable of forming homomeric assemblies, and

seemingly any pairing of subunits compatible with formation of functional heteromeric receptors (4,7) However, several electrophysiological studies failed to find evidence for selective assembly of AMPA receptors, and instead reported findings compatible with unrestricted stochastic association of individual subunits, producing AMPA receptors of variable stoichiometry (3,12).

To clarify whether all combinatorial possibilities of subunit stoichiometries contribute to functional AMPA receptor assemblies, we established an experimental system of quantitative expression of subunit proteins of AMPA receptors in HEK293 cells.

Materials and Methods

Plasmids

Plasmids containing the GluR1 flop (GluR-A flop) and GluR2 flip (GluR-B flip) cDNAs in the pRK5 expression vector under the CMV promoter, which were utilized in these experiments, were kind gifts from Dr. Doris Patneau, and were used with the permission of Dr. Peter Seeburg (Max-Planck-Institute for Medical Research, Heidelberg, Germany).

Plasmid DNA was amplified in DH5α competent cells, purified using the QIAGEN Plasmid Maxi Kit (QIAGEN Sciences, Valencia, CA), and quantified by absorption spectrophotometry. Restriction endonuclease digestion was employed to confirm identification of plasmids.

Transfection of AMPA receptor subunits

HEK293 cells were grown in DMEM with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin at 37°C in 5% CO_2 (all from Gibco-Invitrogen, Carlsbad, CA). The growth medium was replenished every 3 days. Before transfection, HEK293 cells were trypsinized and replated into 100mm culture dishes at a density of~ 6-7 x 10^5 cells/dish. Transient transfection was performed using a polycationic detergent (Lipofectamine/Plus; Invitrogen, Carlsbad, CA), according to the manufacturer's protocol. When nearly confluent, cells were washed twice with DMEM without FBS to completely remove serum. Each transfected plate received a total of 4 μg of the plasmid DNA carrying glutamate receptor cDNAs, with the 2 subunits in varying ratios (1:0, 4:1, 1:1, 1:4, and 0:1), as well as 2 μg per plate of the green fluorescent protein (GFP) expression plasmid pEGFP-C1. Transfected cells were washed with DMEM, trypsinized, and replated on 15mm glass coverslips (1×10^4 cells/per coverslip) for electrophysiological study and on 100mm dishes for protein harvesting. The cells were grown in DMEM with 10% FBS and treated with 10μM 6,7-dinitroquinoxaline-2,3-dione (DNQX). At 1 to 4 days after transfection, cells were selected for patch clamping on the basis of healthy appearance, physical separation from neighboring cells, and detection of green fluorescence under illumination at 480nm.

Western Blotting

Transfected cells were harvested with harvest buffer (1mM EGTA, 1mM EDTA, 1mM phenylmethylsulfonylfluoride, and 100ug/ml aprotinin in phosphate-buffered saline), spun at 13,000RPM for 33min at 4ºC, and sonicated for 5 min. The pellets were resuspended in solubilization buffer (harvest buffer with 1% Triton X-100), mixed for 30min, and repelleted by centrifugation at 13,000RPM for 33 min at 4ºC. The supernatants were transferred to Eppendorf tubes. Protein concentrations were measured by DC protein Assay (Bio-Rad). From each sample, 5 μg of protein was separated by 8% SDS-polyacrylamide gel electrophoresis and transferred to an Immobilon-P polyvinylidene fluoride micropore membrane (Amersham Pharmacia Biotech). The membrane was blocked with Tris-buffered saline (TBS-T; 20 mM Tris-HCl, 137 mM NaCl, pH 7.6, 0.1% Tween 20) containing 5% defatted milk powder at 4ºC for 24 hours, followed by overnight incubation at 4ºC with a polyclonal primary antibody raised against the

C-terminal peptide of GluR1 and GluR2 (Upstate Biotechnology, Lake Placid, NY) diluted 1:2000 in TBS-T with 3% defatted milk powder. Blots were washed three times with TBS-T, incubated with peroxidase-conjugated anti-rabbit secondary antibody in TBS-T for 2 hours at room temperature, rinsed three times with TBS-T, and developed using ECL Western blotting reagents (Amersham Pharmacia Biotech). Blots were digitally imaged with the Fluorchem^{TM} 8800 Digital Imaging System (Alpha Innotech, San Leandro, CA), with adjustment of exposure time to carefully avoid digital signal saturation. Band intensity was quantified as peak area (net of background signal) in line scans of each lane, using ImageQuant software (Molecular Dynamics).

Whole-cell patch-clamp recordings

Whole-cell voltage-clamp recordings of ligand-gated currents were performed according to a previous study (1,10). The borosilicate glass pipettes used had a resistance of 1.8 – 5 M ohms. The intracellular solution consisted of 120 mM CsF, 3 mM $MgCl_2$, 5 mM EGTA, and 10 mM HEPES (pH adjusted to 7.25 with 12 mM CsOH). Seal formation was performed in a buffer containing 145 mM NaCl, 3 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, and 10 mM glucose (pH = 7.40 with NaOH). Cells were accepted for study if a stable seal formed with a whole-cell resistance of at least 120 M ohms and access resistance of less than 10 M ohms.

For determination of AMPA receptor Ca^{2+} permeability, glutamate was applied via a solenoid valve-controlled theta tube applicator, in Na^{+}-free extracellular solutions containing either 15 or 50 mM Ca^{2+}, to which cyclothiazide (50-100μM) was added to inhibit desensitization. The 15 mM Ca^{2+} solution consisted of 12.8 mM $CaCl_2$, 2.2 mM $Ca(OH)_2$, 10 mM glucose, 10 mM HEPES, and 240 mM sucrose, with pH adjusted to 7.4. The 50 mM Ca^{2+} solution consisted of 47.8 mM $CaCl_2$, 2.2 mM $Ca(OH)_2$, 10 mM glucose, 10 mM HEPES, and 147 mM sucrose, again with pH adjusted to 7.4. Extracellular solutions were supplemented with Cd^{2+} (100 μM) to ensure that no contribution from voltage-gated Ca^{2+} channels was possible. For measurement of current rectification, glutamate was applied in a 20mM Na^{+} solution consisting of 15.3 mM NaCl, 4.7 mM NaOH, 2 mM $CaCl_2$, 1mM $MgCl_2$, 10 mM glucose, 10 mM HEPES, and 228 mM sucrose, with pH adjusted to 7.4 with HCl, with tetrodotoxin (0.5 μM) added.

Cells were held at membrane potentials from -110 to +20 mV, and current-voltage (IV) relationships were recorded. Leak current prior to agonist application was subtracted from agonist-evoked peak or steady-state current at each potential. Reversal potentials in 15 mM or 50 mM Ca^{2+} solutions were determined from fit of net peak currents plotted against holding potential. Desensitization was calculated as the difference between the peak current and the current remaining at 800 msec after peak, divided by the peak current. Rectification was calculated as the ratio of currents at +10 mV to those at -80mV, measured in 20 mM Na^{+} solution.

Results

GFP expression was used to identify successfully transfected HEK293 cells. The population of fluorescence-positive cells varied from 5 to 20% of all cells in each experiment (Fig 1). Although we detected AMPA receptor-mediated ion current in every bright fluorescent cell, we could not detect the ion current in cells without fluorescence. This experimental system thus enabled us to choose cells for experiment under fluorescence microscopy.

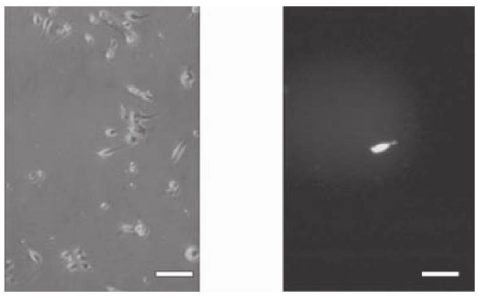

Fig 1
GluR1, GluR2, and green fluorescent protein cDNA-transfected HEK293 cells under bright-field (left) and fluorescence microscopy (right). Bars:50 μm.

We examined AMPA receptor-mediated responses by the whole-cell patch-clamp method in HEK293 cells transfected with GluR1 and GluR2 subunits in varying proportions from 1:0 to 4:1 (termed 1:0, 4:1, 1:1, and 1:4 cells). One of the important assumptions required to interpret the results of experiments is that proportional expression of subunit proteins approximates cDNA transfection ratios. Western blots of samples with co-expression of the GluR1 and GluR2 constructs used in this study indeed showed that relative GluR1 and GluR2 integrated band densities scaled with transfection ratios (Fig 2).

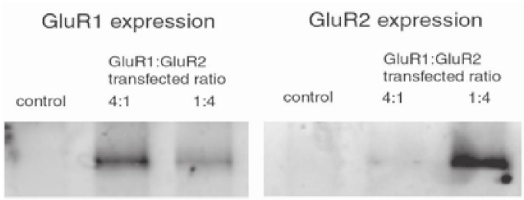

Fig 2
Western blotting of GluR1 (left) and GluR2 (right) proteins in 4:1 and 1:4 transfected HEK293 cells (methods described in text). Control: Cells transfected with GFP alone.

In this study, glutamate (500μM) was applied rapidly in the presence of cyclothiazide, which prevents or slows desensitization of AMPA receptors (9), allowing detection of peak evoked currents at the whole-cell level. In order to sensitively detect changes in divalent cation permeability, currents were measured in Ca^{2+}-based, Na^{+}-free external solutions.

Among the receptors forming from this combination of subunits, three physiological features, Ca^{2+} permeability, rectification, and desensitization, each clearly distinguished AMPA receptors formed from homomeric GluR1 (1:0 cells) from those receptors in which GluR2 predominated (1:1 and 1:4 cells), in both 15 and 50 mM Ca^{2+} conditions.

The 1:0 cells (homomeric GluR1 receptor cells) exhibited strong inward current rectification and relatively positive reversal potentials, due to high Ca^{2+} permeability (Fig 3). The inward currents completely desensitized during 1 sec applications of glutamate, consistent with previous reports of the effects of cyclothiazide (9). These features (inward rectification and high Ca^{2+} permeability) indicate that the receptors expressed in these cells are type II AMPA receptors, as described originally by Iino et al (1990).

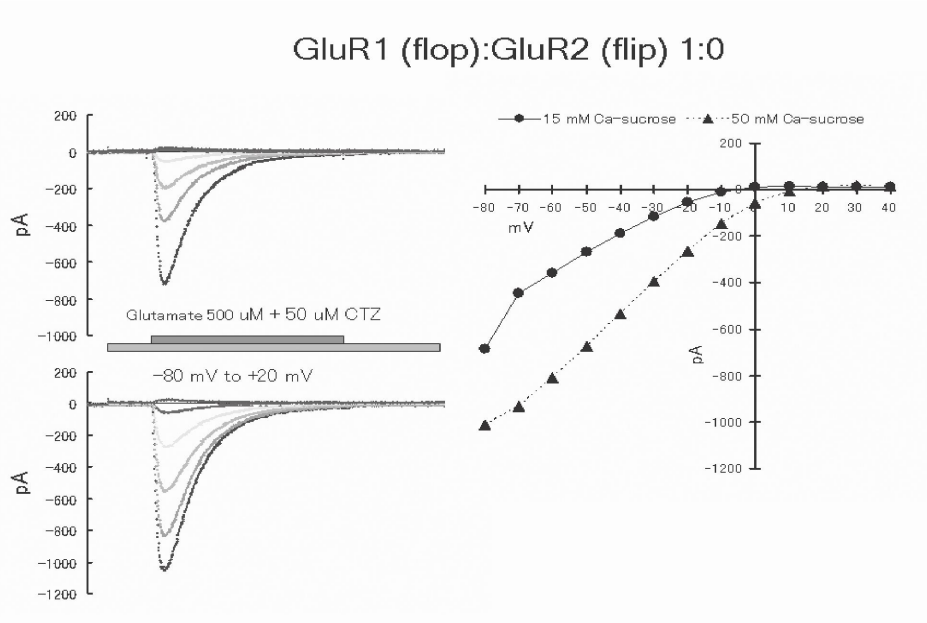

Fig 3
AMPA receptor responses in a 1:0 cell. Whole-cell current responses to 1 sec applications of glutamate (500μM) plus cyclothiazide (50μM) were recorded at membrane potentials ranging from –110 mV to +40 mV. Left: current traces at –80 to 20 mV (20 mV intervals) in 15 (upper) and 50 mM (lower) Ca^{2+}, Na^{+}-free solutions. Right: current-voltage curves, averaged from normalized data to eliminate cell-to-cell differences in current magnitude due to cell size.

In contrast, in 1:1 (Fig 5) and 1:4 cells (Fig 6), the currents evoked by glutamate and cyclothiazide exhibited very little desensitization. The currents were slightly outward-rectifying (due to the high internal Cs^+ concentration) and had uniform left-shifted reversal potentials consistent with low channel divalent cation permeability, features typical of type I AMPA receptors (6,8). Expression of GluR2 alone (0:1 cells) produced only very small evoked currents (not shown), making comparable analysis impossible.

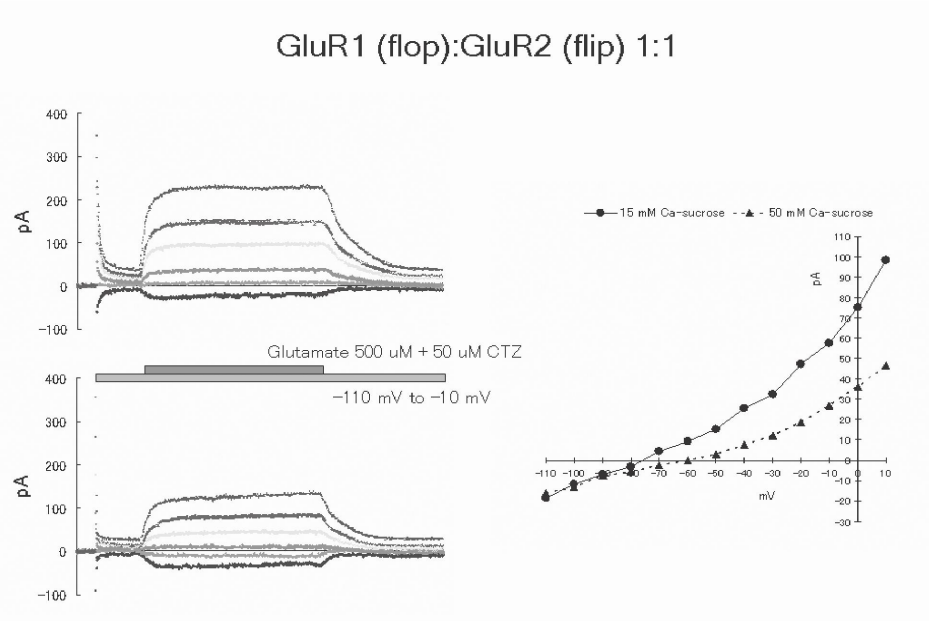

Fig 5
AMPA receptor responses in a 1:1 cell. Whole-cell current responses to 1 sec applications of glutamate (500μM) plus cyclothiazide (50μM) were recorded at membrane potentials ranging from –110 mV to +10 mV. Left: current traces at –110 to -10 mV (20 mV intervals) in 15 (upper) and 50 mM (lower) Ca^{2+}, Na^+-free solutions. Right: current-voltage curves, averaged from normalized data to eliminate cell-to-cell differences in current magnitude due to cell size.

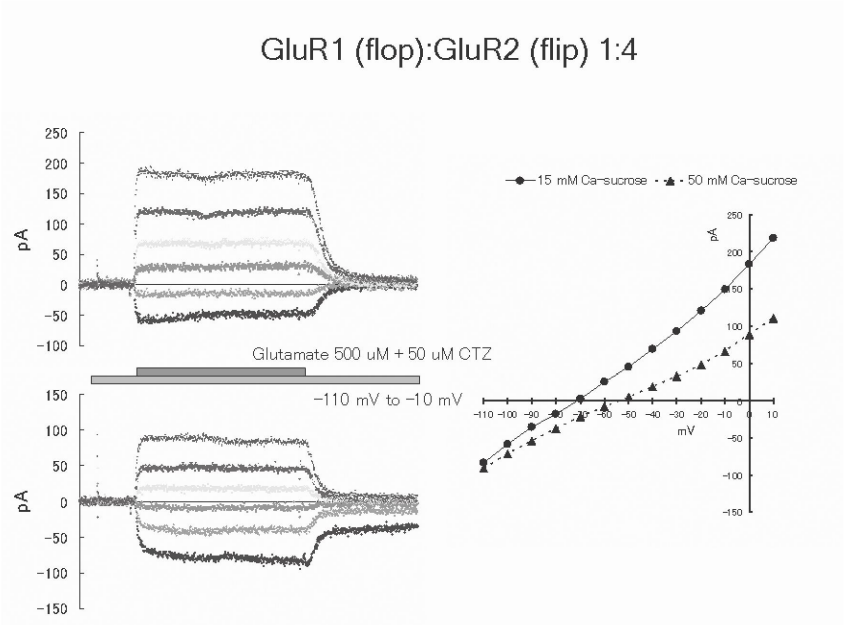

Fig 6
AMPA receptor responses in a 1:4 cell. Whole-cell current responses to 1 sec applications of glutamate (500μM) plus cyclothiazide (50μM) were recorded at membrane potentials ranging from –110 mV to +10 mV. Left: current traces at –110 to -10 mV (20 mV intervals) in 15 (upper) and 50 mM (lower) Ca^{2+}, Na^+-free solutions. Right: current-voltage curves, averaged from normalized data to eliminate cell-to-cell differences in current magnitude due to cell size.

When GluR1 and GluR2 were co-transfected in a 4:1 ratio, the resulting whole-cell current traces exhibited properties that were a mixture of those of type I and type II receptors (Fig 4). Partial desensitization of inward currents could be observed at negative potentials, with outwardly-directed non-desensitizing currents at higher membrane potentials. Current reversal potentials were intermediate between those of type I and type II receptors, consistent with a modest average channel permeability to Ca^{2+}. Interestingly, biphasic currents, with separate contributions of desensitizing inwardly rectified currents and outward non-desensitizing currents, could be seen within individual traces at intermediate potentials, directly demonstrating the presence in one cell of two channel types with different reversal potentials.

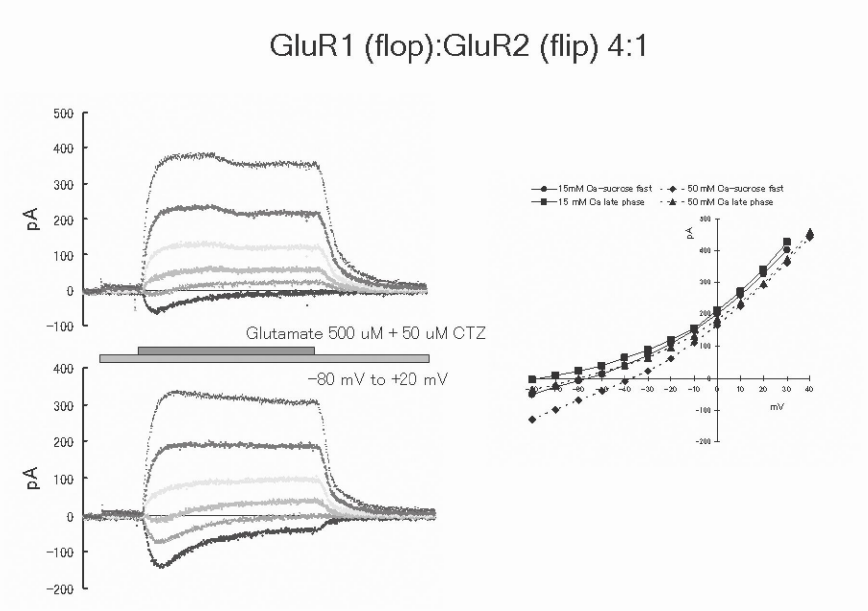

Fig 4
AMPA receptor responses in a 4:1 cell. Whole-cell current responses to 1 sec applications of glutamate (500μM) plus cyclothiazide (50μM) were recorded at membrane potentials ranging from –80 mV to +40 mV. Left: current traces at –80 to 20 mV (20 mV intervals) in 15 (upper) and 50 mM (lower) Ca^{2+}, Na^{+}-free solutions. Right: current-voltage curves, averaged from normalized data to eliminate cell-to-cell differences in current magnitude due to cell size. In these cells, both type I (fast phase) and type II responses (late phase) were observed.

Discussion

The method of co-transfection of GFP and AMPA receptor subunit proteins was useful for detecting successfully transfected cells in the fluorescence microscopy-equipped patch clamp system.

In this study, we performed quantitative transfection of AMPA receptor subunit cDNAs into HEK293 cells. An implicit assumption in this study is that levels of protein subunits expressed reliably reflect cDNA transfection ratios.

Analysis of whole-cell currents generated from co-expression of GluR1 and GluR2 indicated that two functionally distinct receptors are produced, those with strongly desensitizing, inwardly rectifying Ca^{2+}-permeable channels, attributable to GluR1 homomers (type II), and those with non-desensitizing, Ca^{2+}-impermeable heteromeric channels (type I).

With GluR1/GluR2 cDNAs expressed in 1:1 ratio, current traces and current-voltage curves were indistinguishable from those in 1:4 cells, with low reversal potentials and low estimated Ca^{2+} permeability. Thus, these cells had no detectable contributions from Ca^{2+}-permeable, type II currents, despite presumably expressing all possible receptor assemblies including both GluR1 and GluR2 subunits.

In the current traces from cells transfected with a 4:1 ratio of subunit cDNA (4:1 cells), the desensitization properties of GluR1 (flop) homomers provided an independent marker of their contribution. This enabled us to examine whether the whole-cell currents produced when multiple receptor assemblies are expressed could be simply accounted for as the sum of type I and type II

AMPA receptor currents. Individual current traces from 4:1 cells clearly exhibited time-dependent features that could be generated by linear combination of the type I currents (i.e., those from 1:4 cells) and the desensitizing type II currents from 1:0 cells at the same potentials. More generally, the shape of the entire current-voltage relationships could be produced if desensitizing type II receptors and non-desensitizing type I receptors were the only channel types present. The type I response (reversal potential and rectification) of 4:1 cells (the "late-phase response") was indistinguishable from those found in the 1:4 and 1:1 cells, in which GluR2 effects were predominant. Thus, only two functional types of AMPA receptors were detectable in these cells, with desensitization properties segregating closely with the ion-selectivity properties of type I and type II receptors. This suggests that only a single functional type of heteromeric AMPA receptor contributes measurably to whole-cell current. If only a single functional heteromeric receptor is present, it is possible that GluR2 fully determines receptor properties, making heteromeric receptors of all stoichiometries functionally similar. Alternatively, it may be that only one type of heteromeric assembly is expressed.

Further studies for clarifying the stoichiometry of AMPA receptor subunit assemblies are required. The experimental system of quantitative subunit protein expression developed in this study is useful for electrophysiological study of AMPA receptor functions.

Acknowledgment

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