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Single channel recordings of reconstituted AMPA receptors reveal low and high conductance states

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Glutamate receptors belonging to the AMPA (α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid) subclass were partially purified 30- to 60-fold from forebrain of adult rats and incorporated into planar bimolecular lipid membranes. The channel conductance associated with the reconstituted receptors was activated by kainate and AMPA in a manner that suggests cooperative binding of two to three agonist molecules is required to induce channel opening. This conductance was blocked by the specific antagonist DNQX (6,7-dinitroquinoxaline-2,3-dione). When the partially purified AMPA receptors were reconstituted by the tip-dipping method in asymmetric saline conditions ('outside-out configuration'), the addition of 300 nM AMPA to the pseudo-extracellular solution elicited single channel current fluctuations that were also inhibited by DNQX. Analyses of the currents revealed that the ion channels of reconstituted AMPA receptors have two distinct conductance levels of 12 and 60 pS with the great majority of receptors belonging to the former variety. These results suggest that reconstitution may be useful in identifying factors that regulate the binding and conductance properties of AMPA receptors.

Fast excitatory post-synaptic currents at many sites in mammalian telencephalon appear to be mediated by the AMPA (α-amino-3-hydroxy-5-methylisoxazole-4-proprionic acid) subclass of glutamate receptors [3, 6, 14, 16, 18]. Studies of cultured hippocampal neurons have revealed low affinity/high conductance and high affinity/low conductance varieties of such receptors [12, 20, 21]. Both types appear to exhibit agonist cooperativity, voltage independence, and ion selectivity. In accord with this, binding studies have demonstrated the existence of low and high affinity AMPA sites in synaptic membrane fractions prepared from adult rat brain [7, 17, 22]. In addition, binding studies by Hall et al. [7] provided evidence that the two classes of sites represent different states of the same receptor. This study showed that in lysed membranes the number of low affinity sites is far greater than the number of high affinity sites but upon solubilization the balance is reversed. When the loss of receptors during the solubilization procedure is taken into account, the changes in relative numbers of high vs. low affinity receptors are about as expected if the latter were converted into the former [7].

The hypothesis that the AMPA receptor has high affinity/low conductance vs. low affinity/high conductance states predicts that the solubilized receptors (i.e. with a majority of high affinity states) should be predominantly of a low conductance variety. The present experiments used a modified receptor purification procedure and reconstitution in lipid bilayers to test this point and to explore other characteristics of AMPA receptors from adult rat brain.

Receptor purification was accomplished using techniques described elsewhere [1]. Briefly, brains from adult rats were rapidly removed and forebrain membranes prepared using conventional centrifugation techniques. The membranes were solubilized in 1% (w/v) Triton X-100 and then subjected to a sequence of three chromatographic steps in 1% *n*-octylglucoside: (i) DEAE anion exchange, (ii) wheat-germ lectin affinity, and (iii) polyethyleneimine anion-exchange. These steps produced a 30- to 60-fold increase in the number of AMPA binding sites (assayed with [^{3}H]AMPA) per unit protein. A band of 105 kDa corresponding to the expected molecular weight of AMPA receptor subunits [10, 15] was evident

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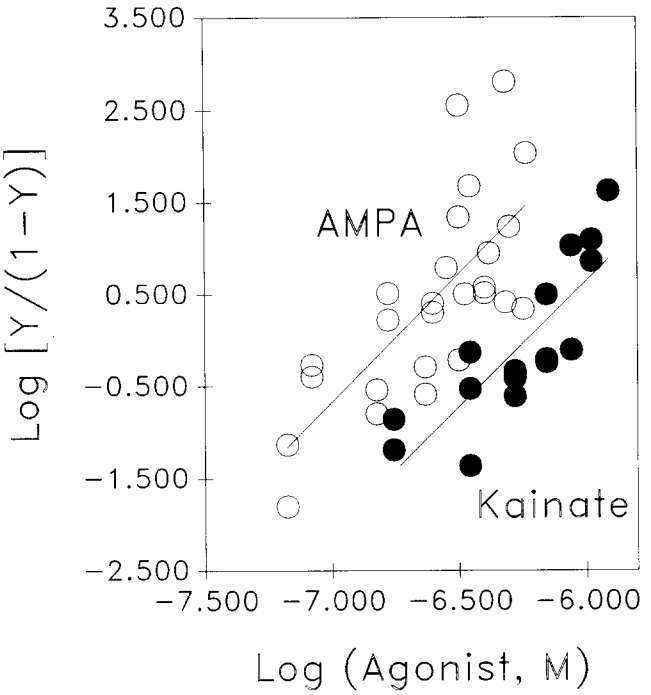

Fig. 1. Dose-conductance relationships for reconstituted receptors activated by AMPA and kainate. Ten μl of a suspension of partially purified AMPA receptor (1 pmol AMPA binding/ml) were added to the *cis*-compartment (3 ml) of the large membrane chamber with stirring. AMPA or kainate was then added to the *cis*-compartment, and membrane current was registered with the voltage clamped at 20 mV. On the *y*-axis, $Y = G/G_{max}$ where G and G_{max} are the conductance values elicited by sub-saturating and saturating concentrations of AMPA or kainate, respectively. The lines were determined by linear regression analysis for AMPA ($r = 0.73$, $P < 0.01$) and kainate ($r = 0.83$, $P < 0.01$) and their respective slopes estimate the number of ligand sites per channel (n_{app}). The AMPA and kainate data have EC_{50} and n_{app} values of 174 nM/2.79 and 580 nM/2.74, respectively (see Table I). The data represent the summary of nine individual experiments similar to that reported in [1].

in polyacrylamide gels of purified material [1] and this band reacted intensely with antibodies against the GluR-1 subunit in immunoblots [1]. These biochemical results indicate that the starting material for the reconstitution experiments contained high concentrations of partially purified AMPA receptors.

Receptors were reconstituted as previously reported in both large (1 mm^2) solvent free [25, 26] and small 'tip-dip' bilayers [24]. The electrical measurement system, assay procedures, and data processing were as described [23]. Bilayers are typically formed from 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine (Avanti Polar Lipids, Inc.) in a buffer composed of (in mM) NaCl 125, KCl 5, NaH_2PO_4 1.25 and Tris-HCl 5, pH 7.4. Patch-bilayers were typically formed in asymmetric saline conditions ('outside-out' configuration) where KCl 110, NaCl 4, $NaHCO_3$ 2, $CaCl_2$ 0.1, $MgCl_2$ 1, MOPS 2 are the mM concentrations inside, and NaCl 125, KCl 5, NaH_2PO_4 1.25, Tris 5 (pH 7.4) are those outside. The bilayer was formed by the successful transfer of two phospholipid monolayers upon the aperture of a bilayer chamber (large membrane) or the tip of a patch pipette (small membrane), after which receptors were added to the bulk solution bathing the *cis* side of the pre-formed bare membrane. After receptor incorporation, AMPA (Tocris Neuramin) or kainate in the absence or presence of the specific antagonist dinitroquinoxaline-2,3-dione (DNQX, Cambridge Research Biochemicals) were applied to the same side of the bilayer with continuous electrical measurements. All patch-clamp data recorded by a VCR system were analyzed off-line. Recorded single-channel events were subjected to computer analysis of amplitude and time distributions. Recorded signals were filtered at 1–5 kHz and sampled at 0.2-, 1-, 5-, or 10-ms intervals, after which they were reduced to series of data sets each containing 7000 data points. The minimum detectable dwell time (0.2 ms) was calibrated by detection of brief events.

The channel conductance associated with the reconstituted AMPA receptors was activated in a dose-dependent manner by AMPA with an EC_{50} of 174 nM and a Hill coefficient of 2.79, and by kainate with an EC_{50} of 580 nM and a Hill coefficient of 2.74 (Fig. 1 and Table I). This suggests that cooperative binding of multiple agonist molecules is required to induce channel opening. Similar cooperativity was evident when the conductance was blocked by DNQX (Table I and Fig. 3C). NMDA and AP-7 (*DL*-2-amino-7-phosphonoheptanoate) caused no significant changes in the conductance of the reconstituted system (data not shown).

When AMPA receptors were reconstituted by the tip-dipping method in asymmetric saline conditions, the addition of 300 nM AMPA to the pseudo-extracellular solution elicited current fluctuations as illustrated in Fig. 2. When the records were analyzed with higher time resolution (Fig. 2B), it appeared that single channel events activated by AMPA consisted of at least two levels of conductance. Fig. 3 shows additional records of single chan-

TABLE I

COOPERATIVITY OF CHANNEL PROPERTIES

Hill plots were used to determine EC_{50} and Hill coefficient values (± one average S.E.M.) for receptor activation by AMPA and kainate (see Fig. 1) and for inhibition of the AMPA (600 μM)-induced activity by DNQX.

Ligand	EC_{50} (nM)	Hill coefficient
AMPA	174 ± 112	2.79 ± 0.54
Kainate	580 ± 240	2.74 ± 0.48
DNQX	440 ± 40	2.77 ± 0.35

nel currents activated by AMPA at holding potentials of −45.8 and −20.5 mV (panels A and B, respectively). These currents were completely inhibited by 1 μM DNQX (Fig. 3C). Amplitude distributions of the current fluctuations were typically skewed to a small extent and contained a small shoulder, fitting a two-level system of relatively small and large conductance amplitudes (Fig. 3D). These levels correspond to a low conductance channel of 11.7 ± 1.6 pS (mean ± S.D.) and a high conductance channel of 60 ± 16 pS, respectively. In the majority of records from patch membranes, the low conductance channel events were much more prevalent than those of high conductance. Finally, Fig. 3E summarizes the results of six individual experiments relating current and voltage, which indicated a small reversal potential of about 6 ± 5 mV.

The properties of the reconstituted receptors correspond well with those described for AMPA receptors found in binding and physiological studies. The EC_{50} value for AMPA (174 nM) is similar to K_d's reported for the high affinity AMPA-binding site measured in membrane and soluble fractions in the absence of thiocyanate ions [8] while the two conductance values obtained for reconstituted receptors agree well with those found in patch-clamp experiments using cultured hippocampal neurons [12, 20, 21]. The receptors were also sensitive to antagonists of the AMPA receptor, voltage independent, unaffected by conditions that block the NMDA class of glutamate receptor, and exhibited reversal potentials for

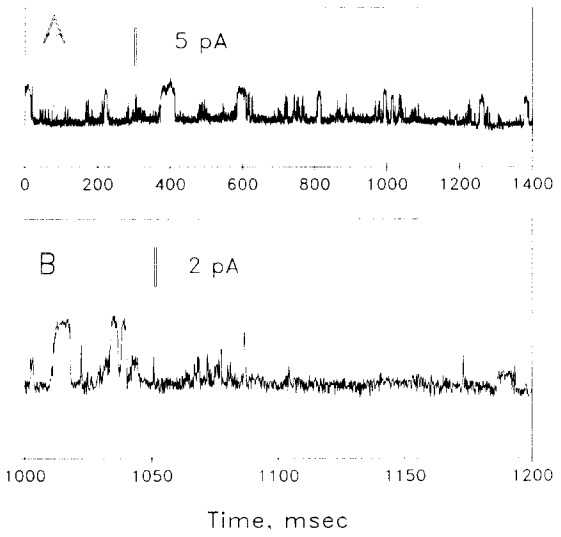

Fig. 2. Single channels activated by AMPA. Receptors were reconstituted in a tip-dip bilayer on the end of a patch electrode. A: upon addition of 300 nM AMPA to the *cis*-compartment, single-channel fluctuations were recorded at −45.8 mV; the signals were subsequently computer-digitized at 0.2-ms intervals after filtering at 1 kHz. Records in the range 1000–1200 ms were expanded in B to show greater time resolution.

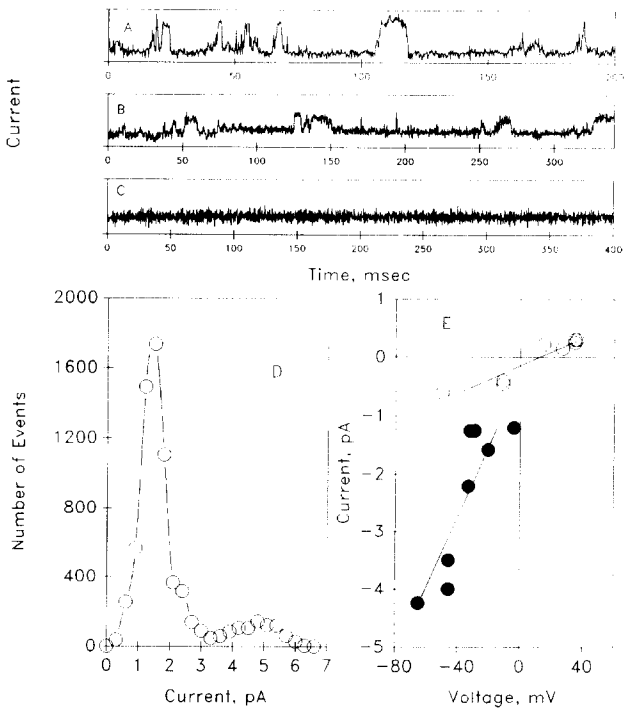

Fig. 3. Single-channel properties of reconstituted AMPA receptors. Receptors were reconstituted in small bilayer patches as in Fig. 2. Single-channel currents were elicited by the addition of 300 nM AMPA to the *cis*-compartment and subsequently recorded and digitized. The bilayer current was measured with the voltage clamped at −45.8 mV (A) and at −20.5 mV in the absence (B) or presence (C) of 1 μM DNQX. *Y*-axis range in panels A, B, and C is 5 pA. The amplitude distribution of single-channel currents in A is shown in D consisting of 7000 points sampled at intervals of 0.2 m, segmented with a bin width of 0.25 pA, and fit with a cubic spline interpolation. The histogram exhibits two maxima at 1.6 pA (channel-closed current level), 4.8 pA (large channel-open current level), and a small shoulder at 2.1 pA (small channel-open current level). The current-voltage relationships for the two maxima populations are shown in E where current amplitude is plotted as a function of membrane voltage using linear regression. Open circles ($r = 0.95$, $P < 0.01$) represent the low conductance channel of 11.7 ± 1.6 pS (mean ± S.D.), and filled circles ($r = 0.86$, $P < 0.01$) correspond to the high conductance channel of 60 ± 16 pS. These data represent the summary of six individual experiments. The data representing small channel currents were taken from records where the low conductance channel events were much more prevalent than those of high conductance.

current flow expected for a ligand-gated cationic channel. It appears then that brain AMPA receptors can be purified and reconstituted in artificial membranes while retaining essential in situ characteristics.

The interaction between agonists and receptors as described by the Hill equation suggests that a significant degree of cooperativity is required to produce channel openings. The cooperativity index obtained for the reconstituted receptors ($n_H = 2.8$) is higher than that typically reported (i.e. $n_H = 1.3$–1.9) for AMPA receptors [5, 10, 11, 20] or for other transmitter receptors [2, 5, 9, 13]. Whether this reflects the use of adult brain for starting

material or is a consequence of purification and reconstitution can only be resolved with further experiments. Recent work indicates that the AMPA receptor is composed of two to five subunits [27] each of which may possess a binding site for agonists [4, 19]. The observed agonist cooperativity in the reconstitution system presumably reflects a requirement for two to three subunits to be occupied in order to trigger a channel event. Alternatively, such cooperativity could also be explained if two subunits whose ligand sites are linked in a cooperative fashion are needed for channel opening. Evidence for cooperativity of this kind has been seen in binding experiments measuring ligand dissociation (unpublished observation). In either case, the existence of such receptors in situ would serve to greatly restrict the number of post-synaptic receptors that generate current upon release of a given amount of transmitter.

As mentioned, previous work [7] showed that solubilization of brain membranes results in an apparent conversion of AMPA-binding sites from a low affinity to a high affinity state. This result led to the suggestion that the high affinity/low conductance and low affinity/high conductance channels observed in physiological experiments represent different functional states of the same receptor and that their properties are sensitive to influences (lipid environment, protein–protein interactions, etc.) present in the synaptic environment. The finding that the great majority of solubilized receptors are of the high affinity/low conductance variety accords with this hypothesis. The purification-reconstitution system described here should thus prove useful for identifying factors that might determine the affinity/conductance states of synaptic AMPA receptors in situ.

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1 Bahr, B.A., Vodyanoy, V., Hall, R.A., Suppiramaniam, V., Kessler, M., Sumikawa, K. and Lynch, G., Functional reconstitution of AMPA-type glutamate receptors from rat brain, J. Neurochemistry, 59 (1992) 1979–1982.

2 Bertrand, D., Ballivet, M. and Rungger, D., Activation and blocking of neuronal nicotinic acetylcholine receptor reconstituted in *Xenopus* oocytes, Proc. Natl. Acad. Sci. USA, 87 (1990) 1993–1997.

3 Blake, J.F., Brown, M.W. and Collingridge, G.L., CNQX blocks acidic amino acid induced depolarizations and synaptic components mediated by non-NMDA receptors in rat hippocampal slices, Neurosci. Lett., 89 (1988) 182–186.

4 Boulter, J., Hollmann, M., O'Shea-Greenfield, A., Hartley, M., Deneris, E., Maron, C. and Heinemann, S., Molecular cloning and functional expression of glutamate receptor subunit genes, Science, 249 (1990) 1033–1035.

5 Curras, M.C. and Dingledine, R., Selectivity of amino acid transmitters acting at *N*-methyl-D-aspartate and amino-3-hydroxy-5-methyl-4-isoxazolepropionate receptors, Mol. Pharmacol., 41 (1992) 520–526.

6 Dooley, D.J. and Bittiger, H., Characterization of neurotransmitter receptors in the rat hippocampal formation, J. Neurochem., 38 (1982) 1621–1625.

7 Hall, R.A., Kessler, M. and Lynch, G., Evidence that high and low affinity AMPA binding sites reflect membrane-dependent states of a single receptor, J. Neurochem., 59 (1992) 1997–2004.

8 Hall, R.A., Massicotte, G., Kessler, M., Lynch, G. and Baudry, M., Thiocyanate equally increases affinity for two AMPA receptor states, Mol. Pharmacol., in press.

9 Heidmann, T. and Changeux, J.-P., Interactions of a fluorescent agonist with the membrane-bound acetylcholine receptor from *Torpedo marmorata* in the millisecond time range: resolution of an 'intermediate' conformational transition and evidence for positive cooperative effects, Biochem. Biophys. Res. Commun., 97 (1980) 889–896.

10 Hollmann, M., O'Shea-Greenfield, A., Rogers, S. and Heinemann, S., Cloning by functional expression of a member of the glutamate receptor family, Nature, 342 (1989) 643–648.

11 Horne, A.L. and Simmonds, M.A., The pharmacology of quisqualate and AMPA in the cerebral cortex of the rat in vitro, Neuropharmacology, 28 (1989) 1113–1118.

12 Jahr, C.E. and Stevens, C.F., Glutamate activates multiple single channel conductances in hippocampal neurons, Nature, 325 (1987) 522–525.

13 Javitt, D.C., Frusciante, M.J. and Zukin, R.G., Rat brain *N*-methyl-D-aspartate receptors require multiple molecules of agonist for activation, Mol. Pharmacol., 37 (1990) 603–607.

14 Kauer, J.A., Malenka, R.C. and Nicoll, R.A., A persistent postsynaptic modification mediates long-term potentiation in the hippocampus, Neuron, 1 (1988) 911–917.

15 Keinanen, K., Wisden, W., Sommer, B., Werner, P., Herb, A., Verdoorn, T.A., Sakmann, B. and Seeburg, P.H., A family of AMPA-selective glutamate receptors, Science, 249 (1990) 556–560.

16 Muller, D., Joly, M. and Lynch, G., Contributions of quisqualate and NMDA receptors to the induction and expression of LTP, Science, 242 (1988) 1694–1697.

17 Olsen, R.W., Szamraj, O. and Houser, C.R., [^{3}H]AMPA binding to glutamate receptor subpopulations in rat brain, Brain Res., 402 (1987) 243–254.

18 Salvaterra, P., Matthews, D.A. and Foders, R., Quantitative relationships of five putative neurotransmitter receptor sites in rat hippocampal formation, J. Neurochem., 33 (1980) 1253–1257.

19 Sommer, B., Keinanen, K., Verdoorn, T.A., Wisden, W., Burnashev, N., Herb, A., Kohler, M., Takagi, T., Sakmann, B. and Seeburg, P.H., Flip and flop: a cell-specific functional switch in glutamate-operated channels of the CNS, Science, 249 (1990) 1580–1585.

20 Tang, C.M., Dichter, M. and Morad, M., Quisqualate activates a rapidly inactivating high conductance ionic channel in hippocampal neurons, Science, 243 (1989) 1474–1477.

21 Tang, C.-M., Shi, Q.-Y., Katchman, A. and Lynch, G., Modulation of the time course of fast EPSCs and glutamate channel kinetics by aniracetam, Science, 254 (1991) 288–290.

22 Terramani, T., Kessler, M., Lynch, G. and Baudry, M., Effects of thiol-reagents on [^{3}H]AMPA binding to rat telencephalic membranes, Mol. Pharmacol., 34 (1988) 117–123.

23 Vodyanoy, V., Cyclic nucleotide-gated electrical activity in olfac-

tory receptors. In J.G. Brand and J.H. Teeter (Eds.), Receptor Events and Transduction Mechanisms in Taste and Olfaction, Marcel Dekker, New York, 1989, pp. 319–345.

24 Vodyanoy, V., Cyclic AMP-sensitive ion channels in olfactory receptor cells, Chem. Senses, 16 (1991) 175–180.

25 Vodyanoy, V., Halverson, P. and Murphy, R.B., Hydrostatic stabilization of solvent free lipid bimolecular membranes, J. Coll. Intern. Sci., 88 (1982) 247–257.

26 Vodyanoy, V., Muller, D., Kramer, K., Lynch, G. and Baudry, M., Functional reconstitution of *N*-methyl-D-aspartate receptors in artificial lipid bilayers, Neurosci. Lett., 81 (1987) 133–138.

27 Wenthold, R.J., Yokotani, N., Doi, K. and Wada, K., Immunochemical characterization of the non-NMDA glutamate receptor using subunit-specific antibodies, J. Biol. Chem., 267 (1992) 501–507.