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Pentameric subunit stoichiometry of a neuronal glutamate receptor

(oligomeric proteins/ion channels/synaptic transmission/signal transduction)

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ABSTRACT **Ionotropic glutamate receptors, neurotransmitter-activated ion channels that mediate excitatory synaptic transmission in the central nervous system, are oligomeric membrane proteins of unknown subunit stoichiometry. To determine the subunit stoichiometry we have used a functional assay based on the blockade of two α-amino-3-hydroxy-5-methyl-4-isoxazole propionate/kainate receptor subunit 1 (GluR1) mutant subunits selectively engineered to exhibit differential sensitivity to the open channel blockers phencyclidine and dizolcipine (MK-801). Coinjection into amphibian oocytes of weakly sensitive with highly sensitive subunit complementary RNAs produces functional heteromeric channels with mixed blocker sensitivities. Increasing the fraction of the highly sensitive subunit augmented the proportion of drug-sensitive receptors. Analysis of the data using a model based on random aggregation of receptor subunits allowed us to determine a pentameric stoichiometry for GluR1. This finding supports the view that a pentameric subunit organization underlies the structure of the neuronal ionotropic glutamate receptor gene family.**

Glutamate, the major excitatory neurotransmitter in the mammalian central nervous system (CNS), and its receptors play an important role in both the biology and pathology of the central nervous system (1–6). According to their modes of action, glutamate receptors are classified as ionotropic receptors (iGluRs) that operate as ligand-gated ion channels, and metabotropic receptors coupled to a G protein-linked inositol phosphate signal-transduction pathway (1, 4–6). iGluRs are subclassified into *N*-methyl-D-aspartate (NMDA) and non-NMDA receptors based on their pharmacological and electrophysiological properties (1, 4–6). Several iGluR subunits have been identified by molecular cloning (4–6). Structurally, iGluRs are thought to be multisubunit assemblies organized around a central ion-conducting pore. Their subunit stoichiometry is unknown (4–7), yet in analogy to the paradigmatic nicotinic acetylcholine receptors (8, 9), they are considered to be pentameric (4–7). Recent findings, however, suggest different protein topologies for iGluRs and acetylcholine receptors (10–14) and raise questions about the actual subunit stoichiometry of iGluRs.

In this study, we have attempted to determine the subunit stoichiometry of homomeric GluR1, an α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA)/kainate receptor subtype. Our strategy involves the use of two GluR1 mutant subunits designed to exhibit different sensitivities to the open-channel blockers phencyclidine (PCP) and dizolcipine (MK-801) (15). Coinjection of different complementary RNA (cRNA) ratios for these GluR1 mutants into *Xenopus* oocytes produces heteromeric receptors with variable sensitivity to the channel blockers. Blocker titration curves for distinct mixtures of heteromeric receptors were best-fitted with a model involving five subunits, consistent with a pentameric subunit stoichiometry for GluR1. A preliminary account of these results was presented elsewhere (16).

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MATERIALS AND METHODS

Site-Directed Mutagenesis and cRNA Synthesis. GluR1 is a cDNA clone encoding a functional AMPA/kainate receptor from human brain (17). Site-directed mutagenesis was carried out using single-stranded DNA or by PCR as described (15). Mutant receptors were confirmed by DNA sequencing. Capped cRNA was synthesized from linearized cDNA using the mMessage mMachine from Ambion (Austin, TX) (18).

Heterologous Expression of Mutant Channels in *Xenopus* Oocytes. Oocytes (stage V and VI) were injected with 50 nl of cRNA (0.2 ng/nl in diethylpyrocarbonate-treated water). cRNA concentration was monitored spectroscopically (18). For a coinjection experiment, the molar fraction for a given species is equivalent to the ratio of its cRNA in the mixture (19). Oocytes were analyzed 3–6 days after injection. Oocytes were transferred to the recording chamber ($V \approx 0.2$ ml) and were perfused (2–4 ml/min) with Ba^{2+}-Ringer's solution [120 mM NaCl/2.8 mM KCl/1.8 mM $BaCl_2$/10 mM TES (*N*-Tris [hydroxymethyl]-2-amino ethanosulfonic acid), pH 7.4] in the absence or presence of 0.5 mM kainate (KA) as the GluR1 agonist. For inhibition experiments, oocytes were challenged with 5-s, 0.5 mM KA pulses in the Ba^{2+}-Ringer's solution supplemented with increased concentrations of the channel blockers (15, 18). KA-evoked whole-cell currents were measured under voltage clamp with a two-microelectrode voltage-clamp amplifier (Turbo TEC O1C; NPI Instruments, Tamm, F.R.G.) (18). Recordings were performed at 19 ± 1°C.

Data Analysis. Curve fitting was done with a nonlinear least-square regression algorithm using the MicroCal ORIGIN version 2.8 (Microcal Amherst, MA) and MATLAB (MathWorks, Natick, MA) software packages. The goodness-of-fit was inferred from the χ^2 test (20). Data are given as mean ± SD with $N \geq 5$, unless otherwise indicated, where N denotes number of experiments.

RESULTS AND DISCUSSION

GluR1 Mutant Subunits Assemble as Heteromeric Receptors. GluR1 receptors are marginally blocked by PCP or MK-801 (15). Point mutations of specific residues in the putative pore-lining M2 segment affect sensitivity to these channel blockers. Replacement of a leucine at position 577 by a tryptophan (L577W) conferred to GluR1 a modest increase in drug sensitivity. As shown in Fig. 1*A* (L577W, *Left*), the KA-evoked ionic current is slightly reduced (≈10%) in the

Abbreviations: iGluR, ionotropic glutamate receptor; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionate; PCP, phencyclidine; MK-801, dizolcipine; GluR1, AMPA/kainate receptor subunit 1; KA, kainate; cRNA, complementary RNA.

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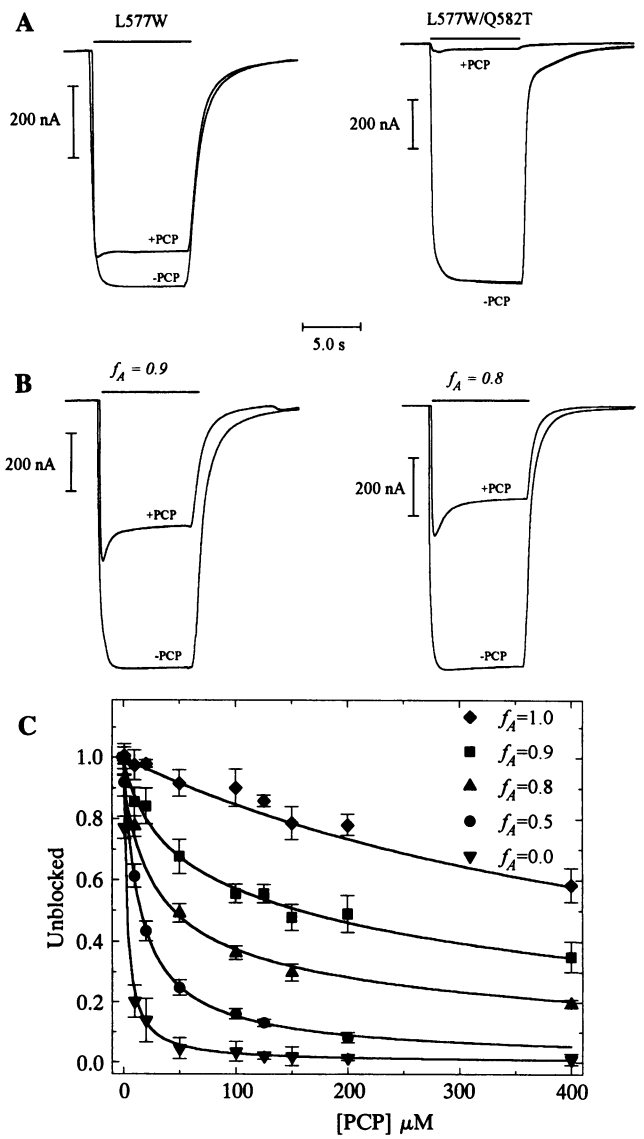

FIG. 1. PCP sensitivity of heteromeric GluR1 receptor channels is determined by the presence of L577W/Q582T mutant subunit. KA-evoked currents from the L577W and L577W/Q582T homomeric (*A*) and heteromeric (*B*) receptor mutants in the absence (−PCP) and presence (+PCP) of 100 μM PCP. Ionic currents were elicited by 0.5 mM KA at V_h = −80 mV in Ba^{2+}-Ringer's solution. Downward deflections represent inward currents. Horizontal bars on top of ionic currents indicate length of KA pulse. (*C*) PCP dose–response curve for mutant receptors was obtained by mixing different cRNA molar ratios of the two types of GluR1 mutant subunits. Values are mean ± SD with $N \geq 8$ oocytes. Solid lines depict the best fit to Eq. **1** using $n = 5$. For $f_A = 0.0$ and 1.0, the equilibrium constants are shown in Table 1. For $f_A = 0.5$, the affinity constants K_i estimated from the model ($i = 0$–5) were 3, 9, 13, 20, 21, and 500 μM. For $f_A = 0.8$ and 0.9, $K_4 = K_3 = 20$ μM; since for these f_A values, the $\Sigma_{i=n-2}^{n} F_i \geq 0.99$ (Eq. **2**) only K_{n-1} and K_{n-2} were considered (21). To properly assess the statistical uncertainty in the estimate of n, the goodness-of-fit was inferred from the χ^2 test considering $4 \leq n \leq 6$ (20). The maximum likelihood of n to match all data is attained with $n = 5$; $n = 5$ is ≥100 times more likely than $n = 4$ and ≈10 times more likely than $n = 6$ to produce the experimental data. Therefore, this analysis implies that $n = 5$ is the minimum number of subunits needed to reproduce all experimental data.

presence of 100 μM PCP. By contrast, a double mutant incorporating Trp-577 and Thr-582 (L577W/Q582T) exhibited a ≈300-fold increase in PCP sensitivity as compared with GluR1 (Fig. 1*A* L577W/Q582T, *Right*) (15). Such a drastic modification of blocker potency appears specific because other functional parameters of the homomeric receptors were not altered significantly. These include the extent and kinetics of functional KA-gated ionic currents, agonist efficacy, ionic selectivity, conductance rectification, and blockade by extracellular divalent cations (Fig. 1*A, Upper*; Table 1) (15). Accordingly, the L577W single mutant is weakly sensitive to blockade by PCP or MK-801 compared with the high sensitivity of L577W/Q582T homomeric receptors.

The extreme relative affinities for drug blockade of the single and the double GluR1 mutants provide an assay to determine the subunit stoichiometry of the oligomeric receptor. For description purposes, the L577W mutant will be hereafter referred to as the insensitive subunit or type A, the L577W/Q582T double mutant as the sensitive subunit or type B, and the respective molar fractions of insensitive and sensitive receptor subunits will be referred to as f_A and f_B. As shown in Fig. 1*B*, coinjection into *Xenopus* oocytes of different cRNA ratios for the insensitive and sensitive receptor subunits produces functional receptors with mixed channel blocker sensitivities. In essence, decreasing the molar fraction of the insensitive subunit with respect to the sensitive one ($f_A = 0.9$, *Left*; $f_A = 0.8$, *Right*) progressively augments the drug potency. The results of a detailed analysis for the PCP blockade are summarized in Fig. 1*C*; data for MK-801 blockade are similar. Notice that for $f_A = 0.5$, the inhibition profile is similar to that displayed by homomeric sensitive receptors ($f_A = 0.0$), suggesting a dominant effect of the drug-sensitive subunit in conferring blocker sensitivity to the assembly. These results indicate that both subunit types coassemble into functional heteromeric receptor channels.

GluR1 Has Pentameric Subunit Stoichiometry. The subunit stoichiometry of the expressed GluR1 oligomers can be determined from blocker titration curves for mixtures of insensitive with sensitive receptors (Fig. 1*C*). We follow the formalism considered by MacKinnon to infer the tetrameric stoichiometry of a recombinant voltage-gated K^+ channel (21). In a coinjection experiment of the type described in Fig. 1 *B* and *C*, homomeric populations of functional channels will contain n subunits of the drug-insensitive monomer (type A), others will have n subunits of the drug-sensitive receptor (type B), and others will be heteromeric ensembles with both types of subunits. For a process of random subunit aggregation the unblocked response, U_{mix}, is given by the following equation:

Table 1. Functional properties of GluR1 mutant receptors

	L577W (Type A)	L577W/Q582T (Type B)
$I_{0.5\ mM\ KA}$, μA	0.2 ≤ I ≤ 2.0	0.2 ≤ I ≤ 2.0
EC_{50KA}, μM	40 ± 3	45 ± 5
I vs. *V*	Inwardly rectifying	Inwardly rectifying
P_{K^+}/P_{Na^+}	1.3 ± 0.1	1.5 ± 0.3
$P_{Ba^{+2}}/P_{Na^+}$	6.1 ± 0.6	4.8 ± 0.2
$P_{Ca^{+2}}/P_{Na^+}$	5.0 ± 0.4	5.2 ± 0.3
K_{PCP}, μM	≈550	2.0 ± 0.2
$K_{MK\text{-}801}$, μM	≈1000	15 ± 2
$K_{Mg^{+2}}$, mM	>1	>1

Homomeric GluR1 mutant receptors were functionally characterized in *Xenopus* oocytes. $I_{0.5\ mM,KA}$ [current evoked by 0.5 mM KA at a holding potential (V_h) of −80 mV]; EC_{50} (concentration of KA required to produce half-maximal response); and K_{PCP}, $K_{MK\text{-}801}$, and $K_{Mg^{2+}}$ (inhibition constants, which correspond to the blocker concentration required to reduce maximal response to one-half) were determined in normal Ba^{2+}-Ringer's solution at V_h = −80 mV as described (15). *I* vs. *V* describes the characteristics of the current-to-voltage relation (15). Relative ionic permeabilities of K^+ (P_{K^+}), Ba^{2+} ($P_{Ba^{2+}}$), and Ca^{2+} ($P_{Ca^{2+}}$) over Na^+ (P_{Na^+}) were as described (A.V.F.-M., W. Sun, and M.M., unpublished work).

$$U_{mix} = \sum_{i=0}^{n} F_i \left(\frac{K_i}{K_i + [\text{blocker}]} \right), \quad [1]$$

where K_i is the inhibition constant for the ith species, F_i is the fraction of channels that are i type, and n is the subunit stoichiometry. Because the coexpression of two different types of subunits follows a binomial distribution (19, 20),

$$F_i = \binom{n}{i} f_A^i f_B^{n-i}. \quad [2]$$

This model is based on two assumptions. (*i*) It considers that protein subunits are expressed to the same extent and aggregate in random order. This condition appears to be met by both mutant GluR1 subunits as evidenced by the similarity of their functional properties, except for the engineered sensitivity to PCP and MK-801 (Table 1). These results suggest a similar global fold for both monomers. Indeed, this is the rationale for using two mutant GluR1 subunits rather than the wild-type receptors, which exhibit different functional properties (15). (*ii*) This analysis implies that a single sensitive subunit confers blocker sensitivity to heteromeric receptors (21). Experimental evidence in support of this constraint is given by the finding that the PCP-unblocked response of heteromeric receptors produced by coinjection of an equal ratio of both subunits is comparable to that exhibited by homomeric sensitive channels (Fig. 1*C*, $f_A = 0.5$ and $f_A = 0.0$). A similar result is obtained from mixing experiments in which the predominant species is the insensitive (type A) subunit. For homomeric insensitive receptors ($f_A = 1.0$), the fraction of the unblocked response, plotted as a function of its product with the PCP concentration, displays a linear relation with a slope corresponding to the reciprocal of the affinity constant (21). At high PCP concentrations, the unblocked response for $f_A = 0.9$ displays a slope that closely corresponds to that of oligomers composed exclusively of the insensitive subunit ($f_A = 1.0$) (data not shown). It follows that the drug-insensitive response represents the contribution of homomeric-insensitive receptors (21).

Having satisfied the basic conditions of the model, we are set to determine n, the subunit stoichiometry of the receptor channel. To find n, Eq. **1** is first transformed as follows:

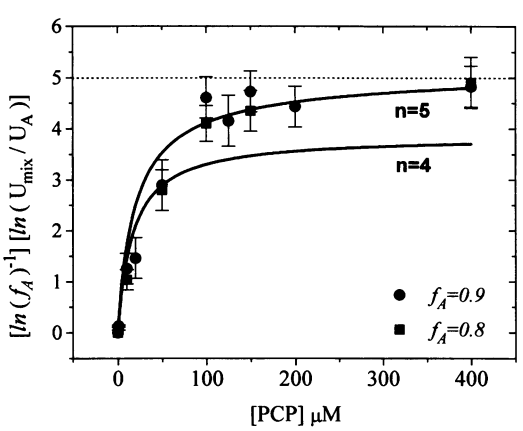

FIG. 2. GluR1 mutant subunits form oligomeric receptors with pentameric subunit stoichiometry. The unblocked responses (Fig. 1*C*) were transformed according to Eq. **3** and plotted as a function of the PCP concentration. At high PCP concentration the data points asymptotically approach a value of ≈5. Solid lines depict the best fit for $f_A = 0.9$, assuming that the equilibrium constant of the species containing at least one drug-sensitive subunit (type B) was 20 μM (Fig. 1*C*). The calculated curve with $n = 5$ approximates the experimental data better than that with $n = 4$. Data are given as mean ± SEM with $N \geq 8$ oocytes, from three independent experiments. Other conditions were as described in Fig. 1.

Table 2. GluR1 channels have a pentameric subunit stoichiometry

	f_A	U_{mix}	$[\ln(f_A)^{-1}][\ln(U_{mix}/U_A)]$
PCP	0.9	0.35 ± 0.02	4.8 ± 0.5
	0.8	0.20 ± 0.02	4.9 ± 0.4
MK-801	0.9	0.30 ± 0.05	4.9 ± 0.6
	0.8	0.19 ± 0.03	4.5 ± 0.6

f_A denotes the molar fraction of drug-insensitive GluR1 mutant subunit in the mixture. U_{mix} represents the unblocked response in a mixing experiment at 400 μM PCP or 1.0 mM MK-801. U_A is the unblocked response of homomeric receptors composed of the drug-insensitive GluR1 subunit and was 0.58 ± 0.01 (mean ± SEM, $n = 16$ oocytes) for 400 μM PCP and 0.50 ± 0.01 for 1.0 mM MK-801 (mean ± SEM, $N = 8$ oocytes). Since at the blocker concentration assayed $R \neq 0$ (Eq. **4**), then $[1/\ln(f_A)][\ln(U_{mix}/U_A)]$ gives an underestimate of n (Eq. **3**).

$$\frac{1}{\ln(f_A)} \ln\left(\frac{U_{mix}}{U_A}\right) = n - \frac{1}{\ln(f_A)} \ln\left(1 - \frac{R}{U_{mix}}\right) \quad [3]$$

with

$$R = \sum_{i=0}^{n-1} F_i \left(\frac{K_i}{K_i + [\text{blocker}]} \right), \quad [4]$$

where U_A denotes the unblocked response for the homomeric drug-insensitive receptors. Notice that when $f_A \rightarrow 1$ and the [blocker] increases, then R asymptotically approaches zero and the left term of Eq. **3** approximates n—i.e.,

$$\frac{1}{\ln(f_A)} \ln\left(\frac{U_{mix}}{U_A}\right) \rightarrow n. \quad [5]$$

Eqs. **3** and **5** give a relation between the observables and the underlying subunit stoichiometry of the functional oligomer. Then, to find the oligomerization number we titrate the unblocked response (U_{mix}) of heteromeric channels created by coinjection of high molar fractions of the drug-insensitive subunit ($f_A \geq 0.80$) (Fig. 1). Transforming data of Fig. 1*C* according to Eq. **3** and plotting them as a function of the PCP concentration produces a saturation curve that asymptotically approaches $n = 5$ (Fig. 2). Indeed, five is the minimum number of subunits that reliably fits all the experimental data as inferred from a goodness-of-fit test (Fig. 1*C*). This finding, therefore, suggests that GluR1 receptors are oligomeric proteins with a pentameric subunit stoichiometry (Table 2). Equivalent results are obtained for MK-801 (Table 2), strengthening the notion of a pentameric structure.

The pore diameter of a recombinant NMDA receptor, a glutamate receptor channel subtype, is 7.3 Å at the entryway and 5.5 Å at its narrowest extent (22), as determined from the cut-off diameter of permeant ions. Similarly, for the γ-aminobutyric acid and glycine receptors, the major inhibitory neurotransmitter receptors in the CNS, the pore constrictions are 5.6 Å and 5.2 Å, respectively (23). The corresponding pore cross sections of 22–24 Å^2 are smaller than that of the AChR (42 Å^2), notwithstanding, they are compatible with a pentameric structure (22–24).

The main consequence of our functional analysis is that neuronal iGluRs have a pentameric structure. That iGluRs are pentameric assemblies supports the tenet of a conserved subunit stoichiometry for members of the neurotransmitter-gated ion channel superfamily, irrespective of the apparently different folding topology of glutamate receptor subunits (8–14, 25–28). This information on the underlying structural organization of iGluRs should help further our understanding of their ion-channel function.

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