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A tetrameric subunit stoichiometry for a glutamate receptor–channel complex

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THE structure of glutamate receptor–channel (GluR) subunits has recently been shown to differ from that of other ligand-gated channels and to contain a voltage-gated channel-like pore-forming motif. The view that the structure of GluR complexes is similar to the pentameric structure of other ligand-gated channels was questioned here. Studies of the response properties of the GluR1 subunit of the AMPA subtype of GluRs, co-expressed in *Xenopus* oocytes with its L646A mutant, which differs only by a greatly reduced sensitivity to quisqualate, provide new evidence suggesting that the GluR1 homomeric receptor channel has a tetrameric structure.

Key words: AMPA; cRNA; Glutamate receptor; Subunit stoichiometry; Tetramer; *Xenopus* oocytes

Introduction

Glutamate receptors (GluRs) mediate most of the excitatory synaptic transduction in the central nervous system. Initially, GluRs were viewed as genuine members of the gene family of ligand-gated channels typified by the nicotinic acetylcholine receptor (nAChR),[1,2] thus forming pentameric structures. Since recent observations have led to the realization that the structure of GluR subunits is very different from that of nAChR but nevertheless contains a potassium channel-like re-entrant loop (reviewed in Refs 3,4), we questioned the suggestion that GluR complexes are formed by the assembly of five subunits.[5–7] By studying the properties of the wild-type (WT) GluR1 subunit of AMPA receptors (AMPA-Rs) co-expressed in *Xenopus* oocytes with the L646A GluR1 mutant, we provide now evidence suggesting that homomeric GluR1 channel complexes have a tetrameric structure. Furthermore, we show that desensitization of a kainate (KA)-activated channel complex requires the interaction of the desensitizing agonist quisqualate (QA) with only one subunit.

Materials and Methods

Oocyte expression: Mutagenesis and oocyte expression were carried out as described previously.[8] For over-expression of WT or mutant GluR1 together with GluR2, WT or mutant GluR1 cRNAs transcribed from pBTG (a vector which gives very high translation rates) were injected together with GluR2 cRNA transcribed from a pSK– (Stratagene, a vector which gives regular expression levels).

Determination of subunit stoichiometry: Two approaches were used: the first was based on the calculation of expected WT fractions, the second on the formalism developed by MacKinnon.[9] For the former, theoretical curves (Fig. 3) were derived from a binomial distribution:

$$F_i = \{n!/[i! \cdot (n-i)!]\} \cdot (f_{mut})^i \cdot (f_{wt})^{(n-i)}$$

where F_i is the fraction of receptor complexes having i mutant subunits, n is the number of subunits in the complex, f_{mut} is the fraction of mutant subunits within the total population and f_{wt} (which equals $1 - f_{mut}$) is the fraction of WT subunits. On the basis of the similarity between the levels of expression of functional WT and mutant receptors when expressed as homo-oligomers or as heteromers with GluR2 (Fig. 1A,B), it is assumed that the co-injection of WT and mutant cRNA into oocytes will result in the formation of hybrid receptors with f_{wt}/f_{mut} ratios equal to the WT/mutant cRNA ratios, allowing the use of cRNA ratios in this calculation. When suggesting that the presence of one WT subunit in the hybrid receptor complexes is enough to confer a WT-like high QA

sensitivity, we assume all the hybrid receptor complexes behave like WT complexes and thus $F(\mathrm{wt}) = 1 - Fn$. When assuming that at least two WT subunits are needed to confer WT-like high QA sensitivity, the expected relationship is $F(\mathrm{wt}) = 1 - [Fn + F(n-1)]$. In both cases, the expected $F(\mathrm{wt})$ values (for the trimeric to hexameric stoichiometries) were calculated for the specific experimental f_{wt} and interpolation was used to generate expected $F(\mathrm{wt})$ curves.

For the second approach, we used the equation developed by MacKinnon:[9]

$$\ln(U_{\mathrm{mix}}/U_{\mathrm{mut}})/\ln(f_{\mathrm{mut}}) = n - 1/\ln(f_{\mathrm{mut}}) \cdot \ln(1 - R/U_{\mathrm{mix}})$$

The responses of each oocyte were used to calculate $\ln(U_{\mathrm{mix}}/U_{\mathrm{mut}})/\ln(f_{\mathrm{mut}})$, where U_{mix} is the fractional response in the oocyte expressing a mixed receptor population at a given QA concentration, U_{mut} is the average fractional response of oocytes expressing only mutant receptors at this particular QA concentration, and f_{mut} is as above. At each QA concentration, these values were averaged for all the analyzed oocytes and plotted against the QA concentration. In order to calculate the expected theoretical values, the right hand side of the above equation was calculated: $n - 1/\ln(f_{\mathrm{mut}}) \times \ln(1 - R/U_{\mathrm{mix}})$ where n is the proposed subunit stoichiometry; f_{mut} is as defined above (weighted according to the number of oocytes from each combination ratio used here); $R = (F0 + F1 + \ldots + Fn - 1) \times \mathrm{IC'}_{50\mathrm{wt}}/(\mathrm{IC'}_{50\mathrm{wt}} + [\mathrm{QA}])$ where $(F0 + F1 + \ldots + Fn - 1)$ is the combined fraction of all the species containing at least one WT subunit according to a binomial distribution (calculated as described above) and $\mathrm{IC'}_{50\mathrm{wt}}$ is the QA IC_{50} value of WT GluR1 corrected according to the KA concentration used:[10] although not simple or pure, the interaction between KA and QA can be closely described as competitive,[10–12] allowing the calculation of $\mathrm{IC'}_{50}$ as described by Patneau and Mayer[10] following Cheng and Prusoff;[13] $U_{\mathrm{mix}} = R + Fn \times \mathrm{IC'}_{50\mathrm{mut}}/(\mathrm{IC'}_{50\mathrm{mut}} + [\mathrm{QA}])$ where Fn is the fraction of complexes containing only mutant subunits and $\mathrm{IC'}_{50\mathrm{mut}}$ is the corrected QA IC_{50} value of the mutant subunit. When [QA] is high, R approaches 0 and the calculated value approaches n.

Results and Discussion

Using site-directed mutagenesis, we have recently identified some of the amino acid residues present at the agonist binding site of GluR1.[8] Monitoring the expression of homomeric GluR1 channels in *Xenopus oocytes* by current measurement under voltage clamp, we have observed that the L646A mutant has most of the properties of the WT GluR1: for equal amounts of injected cRNA, its levels of expression

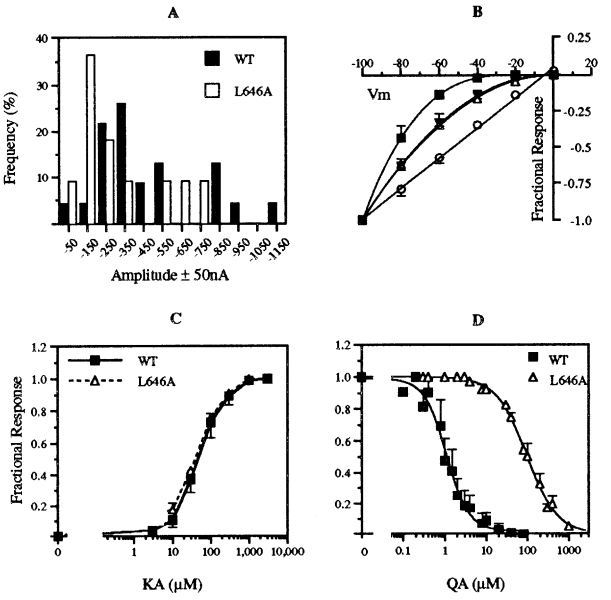

FIG. 1. Characterization of WT and L646A GluR1 expression in oocytes. (**A**) Distribution of the amplitudes of currents evoked by 100 μM KA in oocytes expressing WT GluR1 (solid bars, mean ± s.d.: 470 ± 278; $n = 23$) or L646A GluR1 (hatched bars, 338 ± 238; $n = 11$). The distributions overlap by > 80%, and Student's t-test shows that the difference is not statistically significant. (**B**) Current–voltage relationships in oocytes expressing WT (or L646A mutant) GluR1 alone (rectangles), GluR2 with excess WT GluR1 (triangles, $n = 4$), GluR2 with excess of L646A GluR1 (inverted triangles, $n = 5$) or WT (or L646A) GluR1 with excess of GluR2 (circles). Since the GluR2 AMPA-R subunit imposes a linear I/V relationship on the receptor complexes which include it,[7] over-expression of WT or mutant GluR1 results in a mixed population and partial rectification of the I/V curve which depend on the (homomeric GluR1)/(heteromeric GluR1/GluR2) ratio. An analysis of the statistical deviation of the two curves corresponding to the triangles and inverted triangles indicate that they overlap. (**C**) KA dose–response curves for oocytes expressing WT (rectangles, solid line, $n = 12$) and L646A (triangles, interrupted line, $n = 6$) GluR1. Error bars represent s.d. For WT GluR1, KA $\mathrm{EC}_{50} = 47$ μM and the slope is –1.3. Very similar values are obtained for the L646A GluR1 mutant. (**D**) Inhibition of 100 μM KA-evoked currents upon co-application of QA at increasing concentrations in oocytes expressing WT (rectangles, $n = 24$) and L646A (triangles, $n = 11$) GluR1. Error bars represent s.d. WT GluR1 QA $\mathrm{IC}_{50} = 1.1 \pm 0.3$ μM, slope = –1.6. L646A GluR1 QA $\mathrm{IC}_{50} = 99 \pm 15$ μM, slope = –1.2. An analysis of the statistical deviation of the two slopes indicate that they overlap.

are very similar to those of WT GluR1 (Fig. 1A,B); the WT and L646A GluR1 mutant display the same apparent affinity to KA (Fig. 1C); the KA responses of WT and L646A GluR1 mutant are potentiated to the same extent by saturating concentrations of cyclothiazide (CYZ), a desensitization-attenuating drug,[14,15] indicating that prior to the application of CYZ the equilibrium between the open and desensitized states of KA-activated WT and mutant receptors was the same.[8] Nonetheless, the L646A GluR1 mutant differs from the WT in its QA-induced desensitization properties. These could not be readily observed upon application of the desensitizing agonist QA alone since, in both WT and mutant receptors, hardly quantifiable steady state currents (< 10 nA) were observed. However, a large difference in the sensitivity to QA could be detected, in

the presence of CYZ, as a shift in QA EC_{50} values and a change in the steady state/peak currents ratios[8] or, in the absence of CYZ, in the dose-dependencies of the inhibition by QA of KA-evoked currents, which were found to differ between WT and mutant receptors by a factor of ~100 (Fig. 1D).

The combination of features of WT and of the L646A GluR1 described above provided the necessary conditions to establish the subunit stoichiometry of GluR1 receptor channels on the basis of the rules of a binomial distribution. These require that the WT and mutant L646A GluR1 channel subunits express equally and form mixed oligomeric complexes at random, as demonstrated in Fig. 1A and B.

We therefore co-injected oocytes with various weight ratios of cRNAs encoding the L646A GluR1 mutant and WT GluR1 and studied the QA sensitivity of the expressed channel complexes (Fig. 2). For most of these combinations, a biphasic inhibition can be clearly distinguished. A very good fit of these data could be obtained by using the sum of two logistic equations,[16] revealing the contributions of two receptor populations with distinct IC_{50} values of 1.4 ± 0.3 μM and 81 ± 33 μM QA. As these IC_{50} values are quite similar to those of homomeric WT and L646A mutant GluR1 (1.1 ± 0.3 μM and 99 ± 15 μM QA, respectively, referred hereafter as the parental affinities), the latter values were used in fact to fit the data and yield, as software output, the calculated contribution of each parental population to the hybrid receptor response (solid curve lines in Fig. 2). Attempts to fit the data using free parameters describing an additional intermediate affinity resulted in strong shifts of the affinities attributed to the homomeric WT and L646A mutant GluR1 and in inconsistency between the anticipated contribution of parental and intermediate affinities to the total response, as expected from a binomial distribution according to the fractions of cRNA used, and the actual contribution of each affinity-type, as calculated from the data. We therefore conclude that only the parental QA sensitivities are displayed by the hybrid receptor populations, with no expression of intermediate sensitivities.

Examining the contributions of each receptor population to the observed responses, the relative contribution of the expressed WT phenotype was larger then its original proportion in the cRNA injected (e.g. a 1:5 WT:mutant cRNA ratio exhibits an approximately equal contribution of the parental phenotypes). This indicates that hybrid receptor complexes containing WT and mutant subunits do indeed form and that, in these complexes, the WT high sensitivity to QA is dominant. Figure 3 compares the observed relationship between the proportions of WT cRNA injected and the fractions of receptor populations displaying the WT phenotype to the corresponding theoretical curves expected from the binomial distributions of complexes formed

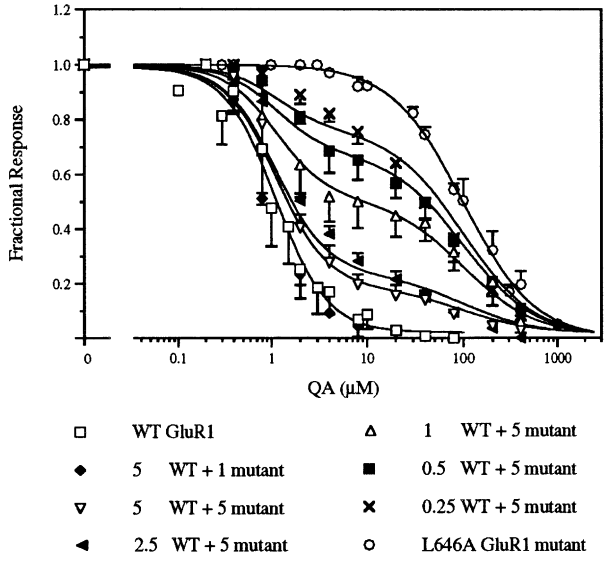

FIG. 2. Inhibition of KA responses by QA at various combination ratios of WT and L646A GluR1 cRNAs. Oocytes injected with combinations of WT and L646A GluR1 cRNAs at the indicated weight ratios were exposed to co-applications of 100 μM KA and increasing concentrations of QA. Error bars represent s.d. The lines show the fit obtained using the sum of two logistic equations[16] with parental IC_{50} values.

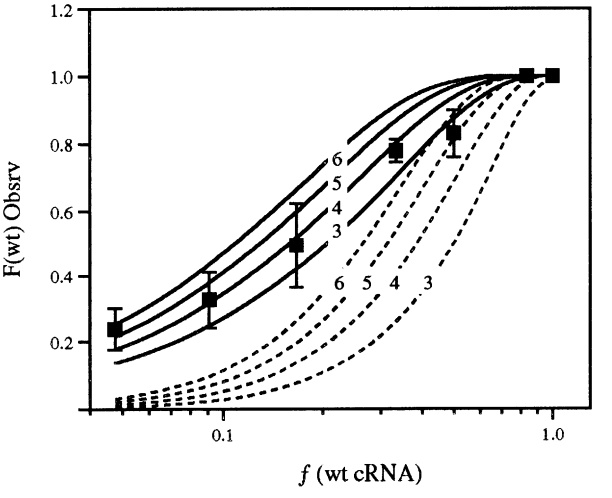

FIG. 3. Analysis of channel stoichiometry: relationship between the fraction of WT cRNA injected and the WT phenotype response observed. The receptor fractions *F*(wt) displaying the WT phenotype were determined as described in Materials and Methods. To account for the s.d. of the experimental points shown in Fig. 2, curves fitting the edges of the s.d. bars were calculated as well, in order to estimate the likely deviation of *F*(wt) (error bars). The lines are theoretical curves describing expected *F*(wt) assuming a binomial distribution of receptor complexes made by $n = 3,4,5,6$ subunits associating at random. Solid lines and interrupted lines correspond to cases in which the WT phenotype of a hybrid receptor complex is due to the presence of one or two WT subunits, respectively.

by the assembly at random of WT and mutant subunits into trimers and up to hexamers. Inspection of Fig. 3 reveals that, regardless of the suggested stoichiometry, the experimental data clearly align with the theoretical curves calculated for the case in which the presence of a single WT subunit in the hybrid receptor has a dominant effect and is enough to account for the high QA sensitivity. Therefore we conclude that the interaction of QA with only one of the receptor subunits is the necessary and sufficient condition for causing the desensitization of a KA-activated oligomeric channel complex. Analysis of the average misfit between the observed WT fractions and the various theoretical values suggests that the data fit best with a tetrameric stoichiometry model.

To determine in an additional fashion the subunit stoichiometry of the GluR1 receptor channel, we further used the sets of equations developed by MacKinnon for the analysis of the subunit stoichiometry of the Shaker potassium channels.[9] Figure 4 presents the results of this analysis for pooled and averaged data from four different combinations of cRNA, along with the theoretical curves for stoichiometries of 3, 4 and 5. At high QA concentrations the curve reaches asymptotically a value of $n = 4$. χ^2 analysis of the set of data, comparing the values expected according to each stoichiometry to the actual values observed for the various QA concentrations, indicates that the tetrameric stoichiometry is ~900 and ~3000 times more likely than the pentameric and trimeric stoichiometries, respectively. We therefore suggest that the GluR1 receptor complex expressed in *Xenopus* oocytes is composed of four subunits.

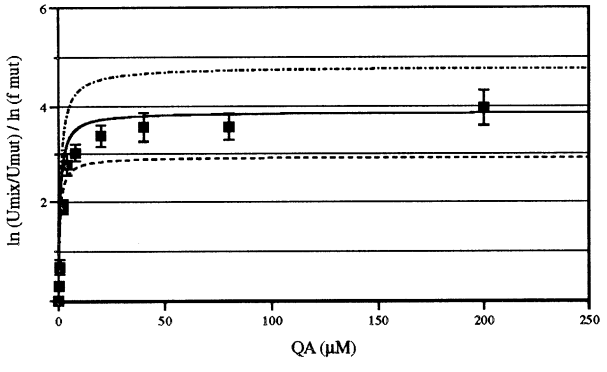

FIG. 4. Analysis of channel stoichiometry: application of MacKinnon's formalism. A plot of $\ln(U_{mix}/U_{mut}) \times \ln(f_{mut})^{-1}$ against QA concentration yields a curve converging to an asymptotic maximum of *n*, the number of subunits in the complex, when the concentration of QA is very high. The data presented on the graph corresponds to four different combination ratios of WT and mutant cRNA. As in all ratios, the curves converged to the same maximum of $n = 4$, the data collected on 29 oocytes were pooled and averaged (± s.e.m.). The lines represent the expected theoretical curves for $n = 3$, 4 and 5.

The limitation common to both methods of analysis is the existence of so far undetected differences between the WT GluR1 and L646A GluR1 mutant, which may lead to their unequal functional expression. This will bias the binomial distribution and preclude the present stoichiometry analysis. In this respect, our present conclusion will remain valid at least as long as the true f_{mut} values do not differ significantly from the set f_{mut} values. The results presented in Fig. 1A,B and C suggest that this condition is likely to be fulfilled. Therefore, on the basis of the results of the two methods used here to analyze subunit stoichiometry, we conclude that a tetrameric stoichiometry is more likely than either the trimeric, pentameric or hexameric stoichiometry. We limit our conclusion to the most probable and simple stoichiometry model but cannot overrule the possible fitting of our data with more complex models.

Beyond the confines of the above mentioned restrictions, we tend to assume that the tetrameric stoichiometry presently suggested for the GluR1 subtype of AMPA-Rs is a feature common to all GluRs, including those expressed in a natural environment. Although some of the biochemical studies that dealt with the determination of the molecular weight of GluRs have favored a pentameric structure, the published data do not rule out a core tetrameric structure, while other even suggest it if considered in conjunction with the proposition that GluRs may interact *in situ* with other proteins which may remain associated with GluRs when the latter are solubilized with weak detergents.[5,6,17,18] Studies of GluR channel properties either do not overrule[7] or suggest[19,20] a tetrameric structure.

Recently, two papers that deal directly with the subunit stoichiometry of AMPA-Rs have reached contradictory conclusions. Ferrer-Montiel and Montal used two GluR1 mutants with amino acid substitutions at the channel pore region and the set of equations developed by MacKinnon[9] to suggest a pentameric stoichiometry for GluR1 receptor channels.[21] However, the mutants tested were not shown to be expressed at the same levels since the amplitudes of their current responses varied up to 10-fold and no data on their distribution and mean values were presented. Furthermore, the channel properties of the two receptor species significantly differ in their bivalent ion permeabilities. This difference leads, according to Lewis's current equation,[22] to unequal amplitudes of current responses recorded from receptor-channels expressed to the same protein level, which may then be further amplified in the oocyte by the secondary activation of Ca^{2+}-dependent Cl^- channels (for comparison, see recording conditions used by Hume *et al.*[23]). Under such circumstances, the fractions of currents recorded

in a mixing experiment are not directly proportional to the ratios of injected cRNAs, leading to an inaccurate estimation of the fraction of the toxin-insensitive species and of the receptor/channel stoichiometry. In the other paper, Wu *et al.*[24] present a variety of compelling biochemical lines of evidence to support the conclusion that AMPA-Rs are formed by a core complex of four subunits.

Conclusion

The question of the stoichiometry of GluRs has been a subject of controversy. We believe that previous biochemical and functional studies[5,17,19,20] and the recent study by Wu *et al.*[24], together with our present data, provide an increasingly strong progression of corroborating evidence for the tetrameric stoichiometry of AMPA-Rs. The presently proposed tetrameric stoichiometry of GluRs provides an additional support to the concept of the existence of an evolutionary link between GluRs and voltage-gated channels.

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