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Assembly Stoichiometry of the GluK2/GluK5 Kainate Receptor Complex

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SUMMARY

Ionotropic glutamate receptors assemble as homo- or heterotetramers. One well-studied heteromeric complex is formed by the kainate receptor subunits GluK2 and GluK5. Retention motifs prevent trafficking of GluK5 homomers to the plasma membrane, but coassembly with GluK2 yields functional heteromeric receptors. Additional control over GluK2/GluK5 assembly seems to be exerted by the amino-terminal domains, which preferentially assemble into heterodimers as isolated domains. However, the stoichiometry of the full-length GluK2/GluK5 receptor complex has yet to be determined, as is the case for all non-NMDA glutamate receptors. Here, we address this question, using a single-molecule imaging technique that enables direct counting of the number of each GluK subunit type in homomeric and heteromeric receptors in the plasma membranes of live cells. We show that GluK2 and GluK5 assemble with 2:2 stoichiometry. This is an important step toward understanding the assembly mechanism, architecture, and functional consequences of heteromer formation in ionotropic glutamate receptors.

INTRODUCTION

Ionotropic glutamate receptors (iGluRs) are key mediators of excitatory synaptic transmission. In mammals, they consist of 18 family members in three main families: AMPA, kainate, and NMDA receptors, which have distinct pharmacology, integrate distinct costimuli, generate unique currents, and have different physiological function (Dingledine et al., 1999; Traynelis et al., 2010). Their diversity is further increased by RNA splicing and editing, coassembly into receptors of mixed subunit composition, and association with several classes of auxiliary proteins, enabling them to perform a wide range of functions at pre-, post-, and extrasynaptic sites (Gereau and Swanson, 2008).

There is a general consensus that all iGluRs assemble as tetramers. While NMDA receptors are obligatory heterotetramers consisting of two glycine- and two glutamate-binding subunits, non-NMDA iGluRs can be formed either by identical or related subunits, although heteromeric assemblies are more common in vivo. Kainate receptor subunits GluK1 (GluR5), GluK2 (GluR6), and GluK3 (GluR7) form functional homomeric receptors, as well as heteromers with one another (Cui and Mayer, 1999). In contrast, the two "high-affinity" subunits of this family, GluK4 (KA1) and GluK5 (KA2), require coassembly with GluK1, GluK2, or GluK3 (Herb et al., 1992; Jaskolski et al., 2005).

One of the best-studied heteromers is the complex between GluK2 and GluK5. GluK2 expression in heterologous cells gives functional homomeric receptors, whereas GluK5 alone is retained in the endoplasmic reticulum (ER) (Gallyas et al., 2003; Hayes et al., 2003; Ma-Högemeier et al., 2010; Ren et al., 2003). Coexpression of GluK5 with GluK2 yields heteromeric GluK2/GluK5 complexes on the cell surface with pharmacological (Herb et al., 1992; Swanson et al., 1998) and functional properties (Barberis et al., 2008; Garcia et al., 1998; Mott et al., 2003; Swanson et al., 2002) distinct from GluK2 homomers. GluK5 is widely expressed in the central nervous system (Herb et al., 1992; Wisden and Seeburg, 1993), and GluK2 is its prevalent interaction partner (Petralia et al., 1994; Wenthold et al., 1994). Accordingly, mice lacking GluK2 show a strongly decreased expression of GluK5 (Ball et al., 2010; Christensen et al., 2004; Nasu-Nishimura et al., 2006). If both high-affinity subunits, GluK4 and GluK5, are missing, kainate receptors no longer contribute to excitatory postsynaptic currents at mossy fiber synapses (Fernandes et al., 2009).

While the GluK2a isoform harbors a forward trafficking motif (Yan et al., 2004), several cytoplasmic ER retention/retrieval motifs and an endocytic motif have been identified in GluK5 (Gallyas et al., 2003; Hayes et al., 2003; Nasu-Nishimura et al., 2006; Ren et al., 2003; Vivithanaporn et al., 2006). Membrane trafficking of GluK5 is only observed in complexes with other subunits like GluK2, which shield or override these motifs. Impairment of the GluK5 motifs yields surface expression of GluK5, but these complexes are nonfunctional (Hayes et al., 2003; Nasu-Nishimura et al., 2006; Ren et al., 2003). Similar mechanisms regulate the trafficking and assembly of other GluK2 isoforms (Coussen et al., 2005), and of other heteromeric complexes (Jaskolski et al., 2005).

Importantly, the finding of intracellular ER retention/retrieval motifs on GluK5, which are overcome upon assembly with a GluK2 subunit, does not answer the question, whether

heteromeric complexes assemble with a defined stoichiometry. This mechanism prevents trafficking of GluK5 homotetramers and, one would expect, also of complexes with three GluK5 subunits, where one GluK2 subunit cannot override the retention motifs, but tetrameric complexes incorporating either one or two GluK5 subunits should be able to reach the cell surface.

Another important factor, which might determine the subunit stoichiometry of GluK2/GluK5 heteromers, is the amino-terminal domain (ATD) of these subunits, which determine the assembly into the distinct families (Ayalon and Stern-Bach, 2001), and whose dimerization is thought to initiate receptor biogenesis (Greger et al., 2007). The recent wealth of structural and thermodynamic data on the isolated ATDs of GluK2 and GluK5 revealed a strong preference for heterodimerization, while homodimerization of GluK2 ATDs was weaker, and of GluK5 ATDs weaker still, leading to a proposal for how 2:2 heteromeric complexes might form (Hansen and Traynelis, 2011; Kumar et al., 2011).

The exact subunit composition of heteromeric receptors at the cell surface, however, is an important, but still missing, piece for understanding the assembly, architecture, and function of non-NMDA iGluRs, which is difficult to deduce from functional experiments. Here, we set out to determine the exact subunit stoichiometry of heteromeric GluK2/GluK5 receptors using a single-molecule subunit counting technique based on photobleaching of fluorescently labeled fusion proteins. This approach enabled us to study the composition of heterogeneous receptor populations on the surface of living cells.

RESULTS

As a first step to investigate the molecular composition of GluK2/GluK5 complexes, we performed single-molecule subunit counting experiments (Ulbrich and Isacoff, 2007) on homomeric GluK2 receptors. Upon fusion of monomeric enhanced green fluorescent protein (mEGFP) to the C terminus of GluK2 (GluK2-mEGFP) and low-density expression in *Xenopus* oocytes, we observed sparse, well-resolved and stationary spots of green fluorescence on the cell surface using total internal reflection fluorescence (TIRF) microscopy (Figure 1A). The photobleaching of a single GFP is a discrete process; thus, the fluorescence intensity of a protein complex with one or several GFP molecules drops in a stepwise fashion, and the number of steps reflects the number of GFP-tagged subunits in the complex. Fluorescence intensity trajectories (e.g., Figure 1B) show that the majority of the GluK2-mEGFP spots bleached in three or four steps, with smaller numbers at one and two steps (Figure 1C, black bars). This distribution of one, two, three, and four bleaching steps originates from the fact that not all subunits contain a fluorescent mEGFP. The distribution observed for GluK2-mEGFP agrees well with the binominal distribution expected for a tetramer, based on a probability of $p = 0.80$ for an individual mEGFP to be fluorescent (Figure 1C, red dashes). Similar values have been obtained on a variety of other membrane proteins (Tombola et al., 2008; Ulbrich and Isacoff, 2007, 2008; Yu et al., 2009), and this value was used to predict distributions throughout this study. The bright fluorescence and immobility of the spots, along with the close agreement between the experimental results and the theoretical

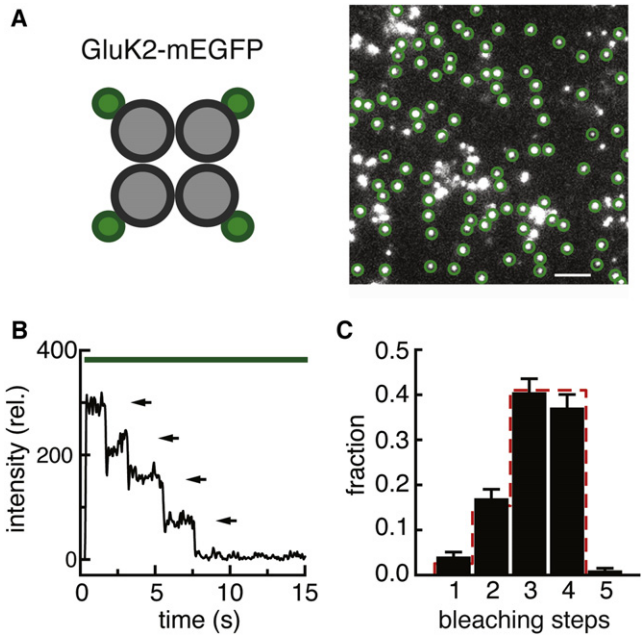

Figure 1. GluK2 Homotetramers

(A) mEGFP was fused to the C terminus of GluK2, expressed in *Xenopus* oocytes and imaged by TIRF spectroscopy. Circles mark single, stationary receptors that satisfy the criteria for analysis. Scale bar: 2 μm.

(B) Fluorescence intensity trace of a representative spot bleaching in four steps indicated by arrows.

(C) Number of bleaching steps observed for a total of 438 spots. The error bars represent the counting uncertainty. The red line gives the binominal distribution expected for a tetramer, based on a probability of 0.80 for an individual mEGFP to be fluorescent.

prediction, demonstrate that the method enables the investigation of the subunit composition of GluK2 containing receptors at the surface of living cells with high accuracy, and confirms that GluK2 forms homotetrameric receptors.

Next, we asked whether GluK5 alone can be detected at the cell surface. In contrast to GluK2-mEGFP, injection of RNA encoding GluK5-mEGFP did not yield bright fluorescent spots as typical for mEGFP-constructs located at the cell surface. Only diffuse dim fluorescence was observed, confirming GluK5-mEGFP expression and being consistent with the expectation that the subunit is retained intracellularly.

The distribution of GluK5 changed when it was coexpressed with GluK2. When GluK2-mEGFP was coexpressed with GluK5-mCherry at an RNA ratio of 1:3 many bright, clearly resolved and immobile red fluorescent spots from GluK5-mCherry were observed (Figure 2A, right image), which colocalized with green fluorescent spots of GluK2-mEGFP (Figure 2A, left image). Indeed, almost all of the red fluorescent spots (95.3%, 341/358) also showed green fluorescence from GluK2-mEGFP. In contrast, a sizable fraction (41.4%, 241/582) of the green fluorescent GluK2-mEGFP spots lacked a red-fluorescent GluK5-mCherry subunit. The results suggest that the cell membrane contained two populations of receptors: GluK2 homotetramers and GluK2/GluK5 heterotetramers. The small