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Coexpression of the receptor-associated protein gephyrin changes the ligand binding affinities of α_2 glycine receptors

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The inhibitory glycine receptor (GlyR) is a ligand-gated chloride channel protein, whose ligand binding α subunit occurs in several isoforms in the mammalian central nervous system. Here we show that coexpression of the GlyR-associated protein gephyrin changes the agonist and antagonist binding affinities of GlyRs generated by α_2 subunit expression in 293 kidney cells. Thus, a receptor-associated protein modifies the functional properties of a neurotransmitter receptor. This may contribute to an optimization of the postsynaptic neurotransmitter response.

Glycine receptor; Heterologous expression; Agonist binding; Gephyrin; Receptor clustering

1. INTRODUCTION

The inhibitory glycine receptor (GlyR) is a chloride channel protein that antagonizes neuronal firing in spinal cord and other regions of the central nervous system [1,2]. The pentameric receptor is composed of homologous ligand binding α and structural β subunits, which arrange into an anion selective transmembrane channel [3–6]. A peripheral membrane protein of 93 kDa has been shown to co-purify with the GlyR [3,7,8] and to be localized at the cytoplasmic face of glycinergic postsynaptic membranes [9,10]. This associated protein, recently named gephyrin [11,12] binds with high affinity to polymerized tubulin [13]. Gephyrin, therefore, has been implicated in the anchoring and/or clustering of GlyRs in the postsynaptic membrane [6,13].

Immunological and molecular cloning data indicate a considerable heterogeneity of GlyR α subunits in the mammalian brain [14–18]. Presently three α subunit variants, α_1–α_3, have been characterized in detail [15–18], which all form glycine-gated chloride channels upon heterologous expression in *Xenopus* oocytes [16–19] or mammalian cell lines [20]. The pharmacology of these homooligomeric GlyRs is very similar to that found for in vivo receptors; however, higher agonist concentrations are required for channel gating. Moreover, in case of neonatal GlyRs, which contain α_2 as the ligand binding subunit [17], significant differences in sensitivity to the glycinergic antagonist strychnine have been revealed by biochemical, electrophysiological and heterologous expression experiments [2,14,16,17]. These discrepancies may relate to the lack of additional subunits [3,5]. Alternatively, other components or modifications specifically occurring in neuronal cells may be required. In this paper we show that coexpression of gephyrin [7–9] changes the ligand binding affinities of α_2 GlyRs. Thus, interactions of neurotransmitter receptors with associated proteins may play a crucial role in shaping postsynaptic receptor properties.

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2. MATERIALS AND METHODS

2.1. *Transfection of 293 kidney cells*

The human GlyR α_2 [16] and the rat GlyR α_3 [18] and β [5] subunit cDNAs as well as the rat gephyrin p1 cDNA [8], each subcloned into the eukaryotic expression vector pCIS, were used to transfect the human embryonic kidney cell line 293 (ATCC CRL1573) by calcium phosphate precipitation as described [20]. For electrophysiological measurements, the cells were grown on fibronectin-coated cover slips (Thermanox) and used for recording 40–50 h after transfection.

2.2. *Western blotting*

Western blot analysis of membrane fractions from the transfected cells was performed with the monoclonal antibody GlyR 5a [4] as described [20].

2.3. *Electrophysiological recordings*

Voltage-clamp recording of membrane currents from 293 cells maintained on coverslips was done as detailed previously using a holding potential of −60 mV [20]. Whole-cell current traces were digitally stored on a personal computer and filtered at 100 Hz before evaluating agonist-induced currents.

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3. RESULTS AND DISCUSSION

To investigate the association of gephyrin with GlyR proteins, we co-transfected 293 kidney cells with human α_2 subunit and gephyrin cDNAs engineered for high-level expression. Both proteins were efficiently synthesized under these conditions as revealed by (i) Western blotting of membrane fractions with mAb5a, a monoclonal antibody selective for gephyrin [4], and (ii) electrophysiological detection of glycine responses in the transfected cells (data not shown). Previous studies have demonstrated that the human GlyR α_2 subunit forms strychnine-sensitive chloride channels upon heterologous expression in *Xenopus* oocytes [16]. Accordingly, large glycine-gated currents were revealed here in co-transfected cells by voltage-clamp recording (Fig. 1). Interestingly, however, the pharmacology of these responses differed from that of the channels formed from the α_2 cDNA alone. Whereas responses to 100 μM glycine in α_2 transfected cells were readily blocked by 1 μM of the high-affinity antagonist strychnine, glycine currents of cells co-transfected with α_2 and gephyrin cDNAs displayed a significantly lower sensitivity to this alkaloid (Fig. 1). Dose–response curves obtained at 100 μM agonist revealed half-maximal inhibition (IC_{50}) values for strychnine of 140 nM for α_2 and 1.8 μM for co-transfected cells, respectively (Table I).

Further analysis of the glycine responses of singly- and co-transfected cells showed that co-expression of gephyrin also produced an increase in agonist affinity. As shown in Fig. 2, the concentration of glycine required for eliciting a half-maximal response decreased from about 80 to 20 μM upon co-transfection of the cells with gephyrin cDNA. Different control experiments showed that the observed changes in agonist

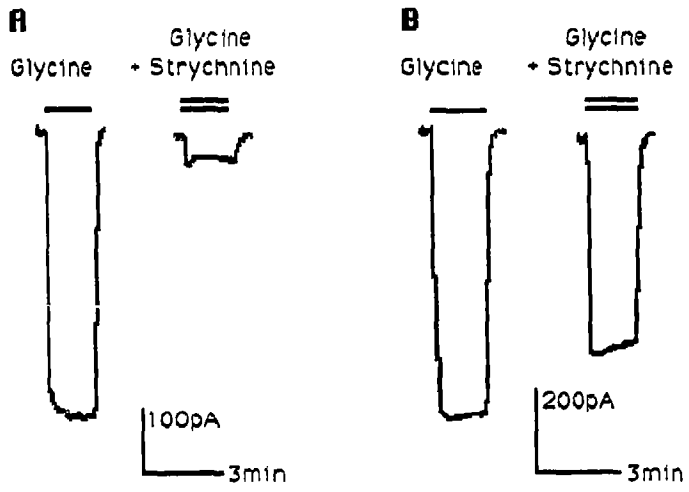

Fig. 1. Strychnine inhibition of glycine responses recorded from 293 cells transfected with either (A) α_2, or (B) both α_2 and gephyrin, cDNAs. Responses to 100 μM glycine were recorded 2 days after transfection in the absence or presence of 1 μM strychnine. Bars indicate the duration of agonist and antagonist application; note different current scales in (A) and (B).

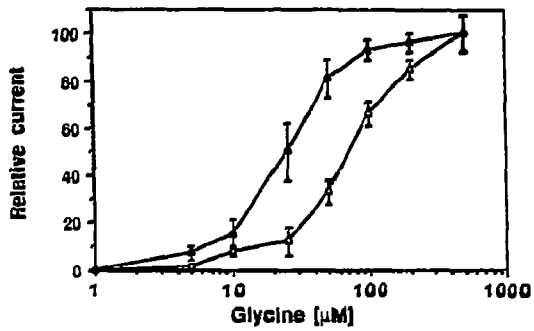

Fig. 2. Glycine dose–response curves obtained from 293 cells transfected with either (○) α_2, or (●) both α_2 and gephyrin, cDNAs. Responses to increasing concentrations of agonist represent mean values from 2–3 independent transfection experiments. Bars indicate SD.

binding were not due to unspecific effects of co-transfection. First, transfection of 293 cells with α_2 and β subunit cDNAs, or with α_3 and gephyrin cDNAs, did not significantly alter the glycine response and strychnine sensitivities of the resulting α_2 and α_3 receptors (Table I). Second, co-transfection of an expression plasmid harbouring a cDNA encoding synaptophysin [21] also did not alter the response of α_2 and α_3 GlyRs (not shown). Thus, the gephyrin protein expressed in this study appears to affect α_2, but not α_3, receptors; this may indicate that different isoforms of gephyrin generated by alternative splicing [8] are differentially associated with the various GlyR isoforms. In conclusion, the pharmacological properties of the GlyR were significantly modified in the presence of gephyrin.

The data presented here indicate that gephyrin alters the pharmacological properties of α_2 GlyRs. Presumably, gephyrin acts by binding to and clustering of receptors, which are otherwise diffusely distributed in the plasma membrane [2,7,8,13]. The hypothesis of enhanced ligand affinity resulting from receptor clustering is supported by recent data from our laboratory, which demonstrate increases in the agonist binding affinity of α_1 GlyRs generated by over-expression in *Xenopus*

Table I

Coexpression of gephyrin alters the pharmacology of α_2, but not α_3, GlyRs

cDNAs transfected	IC_{50} strychnine (nM)	EC_{50} glycine (μM)
α_2	140 ± 43	75 ± 14
α_2 + gephyrin	1800 ± 374	20 ± 3
$\alpha_2 + \beta$	100 ± 21	80 ± 8
α_3	160 ± 8	55 ± 10
α_3 + gephyrin	220 ± 33	65 ± 7

Apparent IC_{50} values for strychnine were determined at a glycine concentration of 100 μM as described [18,19]. EC_{50} values for glycine were estimated from responses obtained with at least 6–7 different concentrations of agonist (5–500 μM).

oocytes (O. Taleb and H. Betz, in preparation). In other words, the dense packing of GlyRs, and possibly other receptors, in the postsynaptic membrane may have functional consequences on the neurotransmitter response. We speculate that inter-receptor cooperatively resulting from lateral interaction of individual receptors [22] may substitute to some extent for intra-receptor cooperativity [19] of agonist binding, thus allowing channel opening at a lower fractional occupancy by the agonist. As a consequence, 'packing' of receptors at postsynaptic membrane domains by association with cytoskeleton binding proteins may represent a central step in the functional maturation of synapses.

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