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Expression of the human glycine receptor α1 subunit in *Xenopus* oocytes: apparent affinities of agonists increase at high receptor density

Omar Taleb[1] and Heinrich Betz[2]

Zentrum für Molekulare Biologie, Universität Heidelberg, Im Neuenheimer Feld 282, D-6900 Heidelberg, Germany

[1]Present address: Laboratoire de Neurobiologie Cellulaire, CNRS – UPR 9009, 5 rue Blaise Pascal, F-67084 Strasbourg, France
[2]Present address: Department of Neurochemistry, Max-Planck-Institute for Brain Research, Deutschordenstrasse 46, D-6000 Frankfurt 71, Germany

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The inhibitory glycine receptor (GlyR) is a ligand-gated chloride channel, which mediates post-synaptic inhibition in spinal cord and other brain regions. Heterologous expression of the ligand binding α subunits of the GlyR generates functional agonist-gated chloride channels that mimic most of the pharmacological properties of the receptor *in vivo*. Here, nuclear injection into *Xenopus* oocytes of a human α1 subunit cDNA, engineered for efficient expression, was used to create GlyR channels over a wide density range, resulting in whole-cell glycine currents of 10 nA to 25 μA. Notably, the pharmacology of these channels changed at high expression levels, with the appearance of a novel receptor subpopulation of 5- to 6-fold higher apparent agonist affinity at current values >4 μA. The low-affinity receptors were readily blocked by nM concentrations of the competitive antagonist strychnine, whereas the high-affinity receptors were more resistant to antagonism by this alkaloid. Picrotoxinin, a chloride channel blocker, inhibited both GlyR populations with equal potency. Our data suggest that receptor interactions, occurring at high receptor density, modify the agonist response of the GlyR. This phenomenon may contribute to neurotransmitter efficacy at fast synapses.
Key words: agonist binding/chloride channel/glycine receptor/strychnine/*Xenopus* oocyte

Introduction

Rapid synaptic transmission in the peripheral and central nervous system (CNS) involves activation of ligand-gated ion channels that transduce neurotransmitter binding into changes of postsynaptic membrane potential (Hille, 1984). Depending on the permeating ion species and its transmembrane gradient, excitation or inhibition of neuronal firing results. The inhibitory glycine receptor (GlyR) is a major postsynaptic chloride channel protein that mediates inhibitory responses in spinal cord and other regions of the central nervous system (Young and Snyder, 1973; Aprison and Daly, 1978; Betz, 1992). Its high affinity for the convulsant alkaloid strychnine has allowed purification of this receptor (Pfeiffer *et al.*, 1982; Graham *et al.*, 1985; Becker *et al.*, 1986). It contains two types of membrane-spanning subunit, of 48 (α) and 58 (β) kDa, in addition to the 93 kDa receptor-associated protein gephyrin (Schmitt *et al.*, 1987; Betz, 1990; Prior *et al.*, 1992). Recently, the primary structures of different α subunit variants and of the β polypeptide have been deduced by cDNA and genomic cloning (Grenningloh *et al.*, 1987, 1990a,b; Betz, 1990; Kuhse *et al.*, 1990a,b, 1991; Agaki *et al.*, 1991; Matzenbach *et al.*, 1994). All share a common transmembrane topology and significant sequence homology with other ligand-gated ion channels, including nicotinic acetylcholine and $GABA_A$ receptor subunits.

Heterologous expression in *Xenopus* oocytes or mammalian cells has shown that GlyR α subunits efficiently assemble into functional agonist-gated chloride channels whose activation is readily blocked by the antagonist strychnine (Schmieden *et al.*, 1989, 1992; Sontheimer *et al.*, 1989; Grenningloh *et al.*, 1990b; Kuhse *et al.*, 1990a,b). Indeed, most of the pharmacological properties of the native GlyR are recovered in these expressed homo-oligomeric receptors, a result that confirms the previous classification (Pfeiffer *et al.*, 1982; Graham *et al.*, 1985; Grenningloh *et al.*, 1987) of α polypeptides as ligand binding receptor subunits. However, a major discrepancy between heterologously expressed GlyRs and those analysed in spinal cord cultures concerns agonist efficacy. Whereas concentrations of 10–100 μM glycine elicit large chloride currents in cultured neurons (Barker and Ransom, 1978; Bormann *et al.*, 1987), EC_{50} values for glycine of the α subunit receptors generated in oocytes range between 0.3 and 1 mM (Grenningloh *et al.*, 1990b; Kuhse *et al.*, 1990a,b; Schmieden *et al.*, 1989, 1992). This cannot be attributed to a lack of other subunits, as injection of poly(A)$^+$ RNA isolated from spinal cord or brain as well as co-expression of the cloned α and β subunits also generate only 'low-affinity' glycine responses in the oocyte expression system (Agaki and Miledi, 1988; Schmieden *et al.*, 1989, 1992; Pribilla *et al.*, 1992). Here we show that the agonist affinity of human α1 subunit GlyRs expressed in *Xenopus* oocytes increases at high expression levels. Our data suggest that interactions between receptors occurring at high density may constitute an important mechanism for optimizing the agonist response of the GlyR, and possibly other ligand-gated ion channels, in the postsynaptic membrane.

Results

α1 cDNA expression in Xenopus oocytes

Synthetic mRNA encoding the α1 subunit of the rat or human GlyR is efficiently translated in *Xenopus laevis* oocytes and assembled into functional glycine-gated chloride channels whose pharmacological properties resemble that of the native receptor in the spinal cord (Schmieden *et al.*, 1989; Grenningloh *et al.*, 1990b). Here, we increased the efficiency of α1 subunit expression by injecting α1 cDNA, engineered for efficient transcription, into the nuclei of the oocytes (Ballivet *et al.*, 1988). This created large current

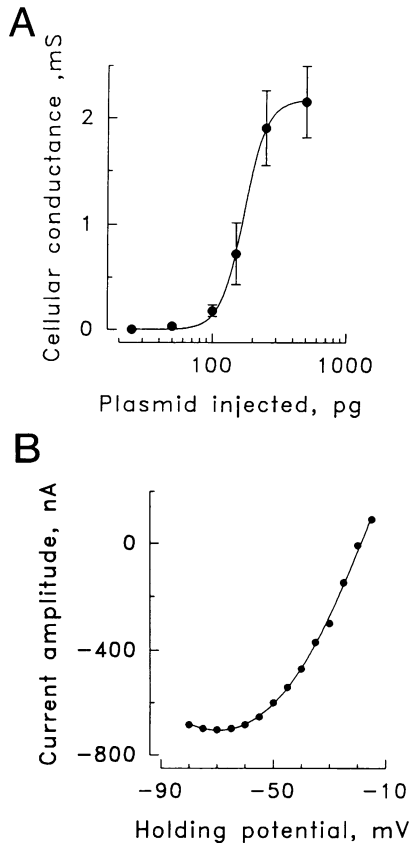

Fig. 1. (**A**) Nuclear injection of GlyR α1 subunit cDNA generates glycine-gated chloride channels in *Xenopus* oocytes: correlation between amount of cDNA injected and glycine current. Plasmid (25–500 pg of DNA) was injected in a constant volume of water (10 nl). Expression was tested at day 4. For each amount of plasmid DNA injected, the cellular conductance was deduced from the slope of the *I/V* curve between −30 and −10 mV obtained with 1 mM glycine. Data are from a single batch of oocytes and are representative of four to seven experiments for each amount of plasmid tested. Bars represent ± SD. Estimated response parameters were G_{max} = 2.172 mS, n = 5 and the amount of DNA eliciting half-maximal expression K = 171 pg. (**B**). Current–voltage relationship of the glycine response at high expression level (oocyte injected with 250 pg of plasmid DNA). The cellular response to 30 μM glycine, within the potential range tested, was well described by the Boltzman equation (continuous curve). Strong rectification was observed at potentials below −50 mV, with an estimated half-inactivation potential of −60 mV. Estimated reversal potential and conductance were −19 mV and 33 μS, respectively.

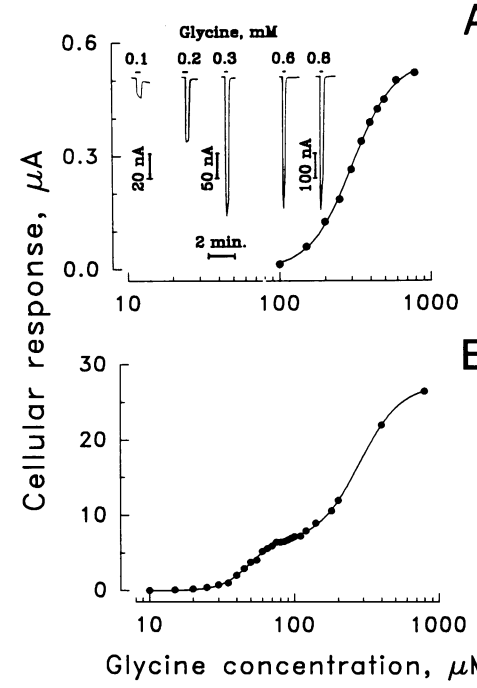

Fig. 2. Glycine dose–response curves at different expression levels. (**A**) Representative dose–response relationship obtained from an oocyte injected with 50 pg of α1 cDNA and clamped at a holding potential of −60 mV. The curve parameters were I_{max} = 551 nA, n = 3.1, and EC_{50} = 304 μM. Original paper traces (inset) illustrate some of the data points. Note that the vertical scale bars are different for the three groups of recordings. Periods of drug application are indicated by horizontal bars. (**B**) Typical glycine dose–response curve obtained upon injection of 250 pg of plasmid. The holding potential was −40 mV. Note the presence of a high-affinity component (I_{max1} = 7.2 μA, n_1 = 4.1 and EC_{50}^1 = 50 μM). The second current component displayed a Hill coefficient and an EC_{50} (I_{max2} = 20.1 μA, n_2 = 3.1 and EC_{50}^2 = 289 μM) comparable with those of the GlyRs generated with 50 pg α1 cDNA.

responses to glycine (Figures 1A and 2), which displayed strong outward rectification at membrane potentials more negative than −50 mV (Figure 1B) and qualitatively resembled the glycine-induced currents recorded from cRNA injected oocytes (Schmieden *et al.*, 1989) or cultured spinal cord neurons (Bormann *et al.*, 1987). At membrane potentials between −30 and 0 mV, the *I/V* curve was nearly linear, resulting in a slope conductance close to that estimated from the Boltzman equation. Figure 1B illustrates a typical current–voltage relationship of the glycine response of an oocyte showing high GlyR expression. Current reversal occurred at a potential of −19 mV. The mean reversal potential obtained from 25 oocytes was −25 ± 4 mV, a value close to the chloride equilibrium potential of *Xenopus* oocytes. Currents up to 15–25 μA were routinely obtained.

The efficiency of cDNA expression was higher than that resulting from cRNA injection into the cytosol: nuclear injection of cDNA produced ~20-fold higher current responses than cRNA expression (Schmieden *et al.*, 1989) in terms of the slope conductance calculated at the linear part of the *I/V* curve (between −30 and −10 mV). Figure 1A shows the latter as a function of the amount of plasmid DNA injected. At saturation (obtained at >500 pg of plasmid), inward currents of up to 25 μA were recorded when applying 1 mM glycine and a 10 mV driving force. The smallest amount of DNA resulting in a detectable current (for a driving force of 20 mV: 22 ± 18 nA; mean ± SD, n = 7) was in the range of 25 pg. Half-maximal expression in terms of GlyR currents was estimated to correspond to an injection of ~170 pg plasmid DNA. Interestingly, the plasmid–response relationship was sigmoidal with a steep slope (Figure 1A), suggesting that a critical α1 subunit concentration has to be reached for efficient formation of functional GlyRs.

Since nuclear injection proved to be highly efficient and allowed variations in glycine-induced cellular conductance over about three orders of magnitude, we performed a more detailed analysis of GlyR α1 subunit receptors using this expression system. Several pharmacological properties were examined, starting with the physiological agonist glycine.

Glycine responses

At low levels of expression (maximal current I_{max} < 1 μA at −40 mV; see Table I), glycine responses were obtained

Table I. Agonist response properties at low and high expression levels

I_{max} (μA)	Glycine				β-alanine				Taurine	
	EC_{50} (μM)		n		EC_{50} (mM)		n		EC_{50} (mM)	n
	a	b	a	b	a	b	a	b		
<1	290 ± 20 (3)		3.2 ± 0.1 (3)		3.6 ± 1.9 (4)		1.2 ± 0.3 (4)		5.6 ± 0.03 (2)	1.2 ± 0.2 (2)
>4	58 ± 8 (7)	280 ± 40 (5)	4.1 ± 0.2 (7)	3.1 ± 0.3 (5)	0.24 ± 0.05 (5)	6.2 ± 2.3 (3)	3.6 ± 0.6 (5)	1.03 ± 0.03 (3)	0.8 ± 0.4 (4)	2.3 ± 0.2 (4)

Agonist dose–response curves were recorded from oocytes injected with 50 pg or 250 pg, of GlyR α1 subunit cDNA, and agonist concentrations for generating a half-maximal response (EC_{50}) were calculated. The data represent mean values ± SD; the number of experiments is given in brackets. a and b refer to the high- and low-affinity components of the agonist-induced currents seen at high expression levels (no separation into high- and low-affinity components was made in case of taurine; see legend to Figure 4). I_{max} refers to maximal glycine current values; n, Hill coefficient.

at agonist concentrations comparable with those required for gating of GlyRs generated from α1 cRNA (Schmieden *et al.*, 1989). As shown in Figure 2A, the threshold glycine concentration for a detectable current (at −60 mV) was typically in the range of 100 μM, and the concentration eliciting a half-maximal response (EC_{50}) ~300 μM. Peak inward currents were dose dependent (inset Figure 2A), and peak amplitudes were distributed along a sigmoid curve. The Hill coefficient (n) for glycine activation was 3.2 (Table I), which is similar to values obtained previously in oocytes (Agaki and Miledi, 1988; Schmieden *et al.*, 1989) and suggests that at least three agonist binding sites have to be occupied for channel opening.

At higher expression levels (I_{max} for glycine >4 μA), a complex dose–response relationship was consistently obtained, which could be best fitted by superposition of two sigmoidal components with different apparent glycine affinities (Figure 2B). Typically, the responses elicited at low agonist concentration had a 5- to 6-fold lower EC_{50} than the second response component (EC_{50} of 50 μM versus 289 μM in the case illustrated). Also, the Hill coefficient of the high-affinity response was 4.1 instead of 3.1 (Table I), suggesting increased cooperativity in channel gating. It is noteworthy that the 'low-affinity' component showed an EC_{50} value similar to that obtained at low plasmid concentrations, although absolute current sizes were very different (Figure 2 and Table I). We therefore interpret the transition from a simple (Figure 2A) to a double sigmoid (Figure 2B) dose–response curve as the appearance of a novel subpopulation of GlyRs with higher agonist affinity.

The increased cooperativity and agonist affinity seen upon high α1 subunit expression may reflect alterations in GlyR subunit stoichiometry or phosphorylation, a dense packing of the receptors in the plasma membrane, or other post-translational modifications occurring under these conditions. In all these cases, a correlation between expression level and the appearance of high-affinity receptors may be expected. Indeed, the maximal high-affinity current (I_{max1}; see Materials and methods) increased with total glycine-activated current ($I_{max1} + I_{max2}$), i.e. the amount of DNA injected (Figure 3). However, even at very high current values, we never obtained a monophasic high-affinity dose–response. Rather, apparent saturation of the high-affinity current was seen at 35 ± 14% (n = 5) of the total response. This saturation occurs before the expression system saturates. Therefore it is unlikely that the low-affinity GlyRs represent heteromeric receptors of the α1 subunit and some hypothetical subunit endogenously present in the *Xenopus* oocyte; accordingly, the high-affinity GlyR should then be a homomeric receptor, which appears after titration of this hypothetical endogenous subunit by the α1 GlyR polypeptide. Rather, these data reinforce the conclusion that high-affinity GlyRs appear at high GlyR density and suggest that factors other than α1 subunit concentration limit their appearance.

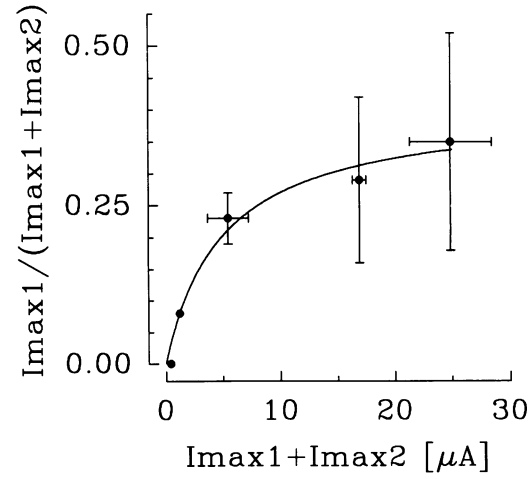

Fig. 3. Appearance of high-affinity α1 GlyRs. The relative fraction of the high-affinity component [$I_{max1}/(I_{max1} + I_{max2})$] of the dose–response curve is shown as a function of the expression level, i.e. the maximal estimated current ($I_{max1} + I_{max2}$). Data points are mean values from two to six experiments, and bars represent ± SD. The data are well described (R^2 = 97.62%) by a hyperbolic equation, with a calculated half-saturation constant of 5 μA. Saturation of high-affinity GlyR formation was obtained at ~40% of the maximal current.

Sensitivity to other agonists

The amino acids β-alanine and taurine are also effective GlyR agonists. Here, β-alanine and taurine responses of oocytes injected with α1 subunit cDNA were examined initially under conditions of low receptor expression (I_{max} for glycine <1 μA). As reported previously for α1 cRNA expression (Schmieden *et al.*, 1992), EC_{50} values and Hill coefficients of 3.6 mM and n = 1.2 for β-alanine, and 5.6 mM and n = 1.2 for taurine, were obtained (Figure 4A and Table I).

Upon injection of large amounts of α1 cDNA, significant increases in β-alanine and taurine affinity were observed. For β-alanine, a clearly biphasic dose–response was seen with mean EC_{50} values of 0.24 and 6.2 mM, respectively. This is very similar to the biphasic response obtained with

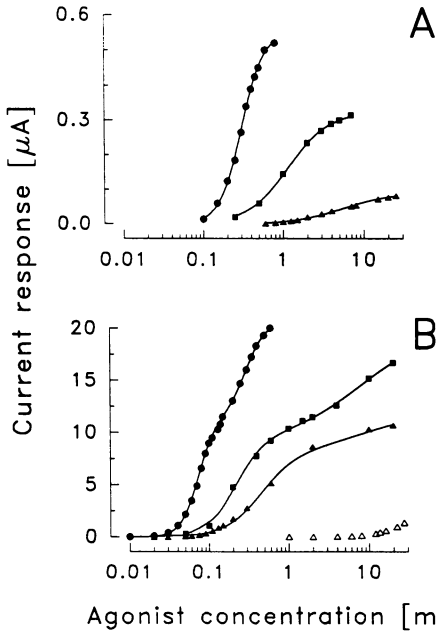

Fig. 4. Responses to different GlyR agonists. (**A**) Dose–response relationships obtained from an oocyte injected with 50 pg of α1 cDNA. The holding potential was −60 mV. Response parameters for glycine (●), β-alanine (■) and taurine (▲) were I_{max} = 551, 325 and 91 nA, n = 3.1, 1.3 and 1.1, and K = 304, 1158 and 5566 μM, respectively. (**B**) Dose–responses from oocytes expressing high GlyR levels (250 pg of plasmid injected) and recorded at a holding potential of −40 mV. Note biphasic curves for glycine and β-alanine. For taurine, no clear separation into two response components was obtained. However, the data were fitted best using a two-components model (see Materials and methods). In contrast to (A) GABA (△) also produced a current response [~3 μA at the highest concentration (27 mM) tested]; the Hill coefficient for GABA determined by log-probit plots was n = 2. Dose–response parameters were for glycine I_{max1} = 10.78 μA, n_1 = 3.9, K_1 = 72 μM, I_{max2} = 10.14 μA, n_2 = 3.1 and K_2 = 290 μM, for β-alanine I_{max1} = 9.42 μA, n = 2.3, K_1 = 218 μM, I_{max2} = 10.13 μA, n_2 = 1.0 and K_2 = 7.89 mM and for taurine I_{max1} = 7.5 μA, n = 2.1 and K_1 = 450 μM. In the case of the taurine response, we assumed that a second current component contaminates the plateau of the high-affinity current; estimated parameters for this low-affinity current were I_{max2} = 5 μA, n = 1 and K_2 = 9 mM.

glycine. For taurine, again a shift in dose–response towards high-affinity was obtained (Figure 4); however, in the latter case two current components could not be separated. On average, affinity increases were 15- and 7-fold for β-alanine and taurine, respectively (Table I). Interestingly, GABA which at low expression levels did not generate any detectable current even at 20 mM, produced a consistent response in cells (n = 10) displaying large maximal glycine currents, when applied at concentrations ≥1 mM (Figure 4B). At the highest concentration used (27 mM), GABA evoked a current of 3 μA (Figure 3B), which corresponds to ~15% of the glycine I_{max} recorded on the same cell.

Concomitant with the observed decreases in EC_{50} values, the Hill coefficients for the high affinity β-alanine and the taurine responses also changed, and values of up to 3.6 and 2.3 were found, as compared with 1.2 at low expression levels (Table I). Thus, agonist cooperativity increased at high expression levels for all GlyR agonists tested.

Strychnine inhibition

The alkaloid strychnine is the most selective GlyR antagonist known to compete with glycine binding (Becker *et al.*, 1986),

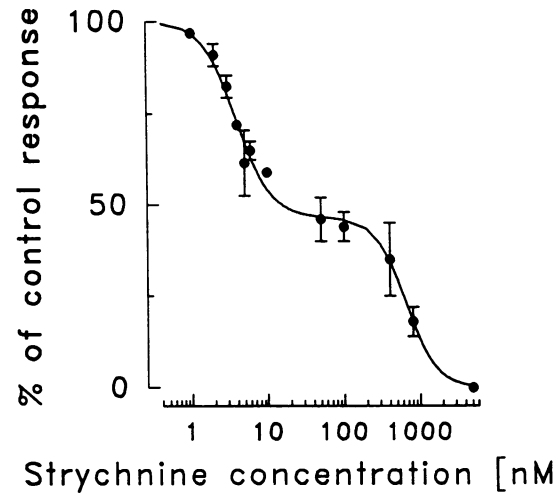

Fig. 5. Strychnine inhibition of agonist responses of oocytes injected with 250 pg of the α1 cDNA. Note biphasic inhibition of the current induced by 300 μM glycine. Data points are from two to four experiments; error bars represent ± SD (when larger than symbol). The continuous curve was fitted (R^2 = 99.17%) as described in Materials and methods. Estimated IC_{50} values and Hill coefficients were 4 nM and n = 2.0 for the first current component, which corresponds to 53% of the total current, and 650 nM and n = 2.2 for the second component, respectively.

whereas picrotoxinin, a potent chloride channel blocker (Barker *et al.*, 1983), inhibits both α1 GlyR and $GABA_A$ receptor chloride channels (Betz, 1992). Here, picrotoxinin blocked glycine responses with an IC_{50} of ~1 μM at all expression levels tested (data not shown; but see Schmieden *et al.*, 1989; Pribilla *et al.*, 1992; Bormann *et al.*, 1993), whereas strychnine exhibited different antagonist efficacies depending on the presence of the high-affinity response. In oocytes injected with low amounts of α1 cDNA, strychnine antagonized glycine responses with a K_i of 12.9 ± 3.2 nM (n = 3), a value similar to that reported for native and heterologously expressed GlyRs (Pfeiffer *et al.*, 1982; Schmieden *et al.*, 1989, 1992; Sontheimer *et al.*, 1989; Betz, 1992). At high current values, however, ~50% of the current was inhibited with a K_i of ~2 nM (Figure 5). The remaining response exhibited a considerably lower sensitivity, with half-maximal inhibition being seen at ~650 nM strychnine which corresponds to a calculated K_i value of 106 nM. We conclude that high-affinity GlyRs display reduced strychnine sensitivity.

Discussion

In this study, we used DNA injection into *Xenopus* oocyte nuclei (Ballivet *et al.*, 1988) to achieve an efficient expression of human α1 subunit GlyRs. Our data confirm that expression by nuclear injection of cDNA, engineered for efficient transcription, is indeed more efficient than cRNA injection into the cytosol. This probably reflects both the high efficacy of the cellular transcription machinery and the lack of degradation normally observed with RNA injected in the cytoplasmic compartment. Nuclear injection allowed formation of functional GlyRs over a wide range of expression levels, with a steep increase in current as higher amounts of DNA were injected. This apparently cooperative increase in glycine current presumably reflects a critical dependence of GlyR assembly on subunit concentration.

The efficient expression of α1 receptors allowed the demonstration of a novel GlyR subpopulation, which appears at high expression levels and displays an increased affinity for gating by glycine and other agonists. This 'high-affinity' GlyR is selective for chloride ions and readily blocked by picrotoxinin with an IC_{50} value very similar to that found for 'low-affinity' α1 GlyRs generated from cRNA (Schmieden *et al.*, 1989; Pribilla *et al.*, 1992) or low amounts of cDNA (this study). We therefore assume that the bore of the chloride channel is identical in both types of receptors and, hence, the same pentameric structure (Langosch *et al.*, 1988; Kuhse *et al.*, 1993) likely to be conserved at all expression levels tested. Biochemical and single channel data on native and/or recombinant GlyRs are consistent with this interpretation (Langosch *et al.*, 1988; Sontheimer *et al.*, 1989; Bormann *et al.*, 1993). We therefore propose that the appearance of GlyRs with high agonist affinity is a consequence of high receptor density in the plasma membrane.

How may changes in receptor density affect agonist binding? First, overexpression could saturate post-translational modification reactions which regulate receptor properties. Phosphorylation by a cAMP-dependent kinase increases glycine responses in cultured neurons (Song and Huang, 1990); however, the human α1 subunit does not contain a good consensus phosphorylation site (Grenningloh *et al.*, 1990b). Also, the extent of receptor glycosylation may decrease upon injection of high amounts of α1 cDNA; but in spinal cord cultures inhibition of *N*-glycosylation by tunicamyin reduces the number of functional GlyRs (W.Hoch, F.Holzinger, H.Betz and C.M.Becker, in preparation). We therefore suggest a second interpretation, which relates the affinity changes observed to inter-receptor cooperativity.

Cooperativity in agonst binding has been extensively demonstrated for different neurotransmitter receptors in excitable membranes. In the case of the nicotinic acetylcholine receptor, open probabilities drastically increase upon occupation of its two agonist binding sites (Colquhoun and Sakmann, 1985). Gating of GlyRs and $GABA_A$ receptors in cultured neurons similarly displays Hill coefficients of ≥ 2.0 (Bormann *et al.*, 1987). From theoretical considerations, it has been suggested that binding of more than one agonist molecule is required to provide sufficient energy for channel activation (Jackson, 1989). Consistent with this view, the low-affinity α1 subunit GlyR investigated here also showed Hill coefficients of ≥ 3.0 for gating by glycine (Schmieden *et al.*, 1989, 1992; and this study). Importantly however, not only the apparent affinities, but also the Hill coefficients of all agonists tested, increased when high amounts (250 pg) of α1 cDNA were injected. Therefore, the behaviour of the high-affinity GlyRs cannot be explained by a model in which intrareceptor cooperativity of agonist binding decreases at high expression levels, and thus allows gating at lower agonist occupancy. Rather, our data are consistent with positive interactions between individual GlyRs. Accordingly, a high density of GlyRs in the plasma membrane is proposed to favour lateral allosteric coupling between single channel molecules, and thus to compensate for submaximal agonist occupation. In other words, packing of individual GlyRs into a larger supramolecular structure is suggested to alter the gating requirements of the integral chloride channel.

The concept of inter-molecular cooperativity in biological membranes is not novel, but was proposed >20 years ago (Changeux *et al.*, 1967). Different investigators have shown that the biophysical and pharmacological properties of membrane proteins can change at high density. In reconstitution experiments with purified preparations of nicotinic acetylcholine receptors (Schindler *et al.*, 1984) and voltage-gated Na^+ (Boheim *et al.*, 1985) and Ca^{2+} (Hymel *et al.*, 1988) channels, the gating and/or conductance properties of these channel proteins were found to be altered at high protein:lipid ratios. Furthermore, voltage-gated K^+ channel cRNAs have recently been shown to generate K^+ currents of distinct biophysical and pharmacological properties upon high and low level expression in *Xenopus* oocytes (Guillemare *et al.*, 1992; Honoré *et al.*, 1992). Finally, statistical evaluations of single channel recordings from muscle cell cultures are suggestive of cooperative interactions between nicotinic acetylcholine receptors in a native plasma membrane (Yeramian *et al.*, 1986). We therefore conclude that the apparent agonist affinity increase observed in our recordings is likely to reflect a general tendency of membrane proteins to interact allosterically at high packing density.

Different experimental observations support the idea that 'high-affinity' GlyRs are cooperatively coupled in the oocyte membrane. First, the Hill coefficient for agonist activation increased at high receptor density not only for glycine, but also for β-alanine and taurine, two agonists which display Hill coefficients close to 1.0 at the low affinity α1 GlyR. Second, the strychnine sensitivity of glycine responses also changed at high expression levels. Alkaloid inhibition of the high-affinity current required much higher strychnine concentrations than antagonism of the low-affinity receptor population. This is consistent with a lower agonist occupancy of the individual channels being sufficient for gating of cooperatively coupled GlyRs.

Physiological implications

The observations made in this study are based on the heterologous expression of a homo-oligomeric GlyR. The question thus arises of whether our findings simply reflect an artifactual situation or may also be relevant for receptor activation *in vivo*. Several arguments can be raised in favour of the latter view. First, different lines of evidence suggest the existence of homo-oligomeric GlyRs in the mammalian CNS: (i) biochemical data on mouse embryonic spinal neurons in culture indicate a homomeric structure of the neonatal α2 GlyR isoform (Becker *et al.*, 1989); (ii) under our experimental conditions the assembly of functional α1 subunit GlyRs was at least 2000-fold more efficient than that of homomeric $GABA_A$ receptor complexes (O.Taleb, unpublished data); and (iii) picrotoxinin-sensitive glycine responses indicative of homomeric GlyRs (Pribilla *et al.*, 1992) have been detected in several regions of the mammalian CNS (see Becker *et al.*, 1992). The natural occurrence of homo-oligomeric GlyR channels may thus be anticipated. Second, both electrophysiological and immuno-electronmicroscopical data on the goldfish Mauthner cell (Faber *et al.*, 1985; Triller *et al.*, 1990) and rat central neurons (Triller *et al.*, 1985; Altschuler *et al.*, 1986) clearly show that high GlyR densities are reached at the synaptic cleft. A GlyR-associated 93 kDa protein, gephyrin (Schmitt *et al.*, 1987; Prior *et al.*, 1992), is also highly concentrated at glycinergic post-synaptic membrane specializations (Triller

et al., 1985; Altschuler *et al.*, 1986) and binds, *in vitro*, with high affinity to tubulin (Kirsch *et al.*, 1991). This protein has recently been shown to be essential for the formation of the post-synaptic receptor matrix (Kirsch *et al.*, 1993). Interestingly, co-expression of gephyrin increases the glycine affinity of α2 subunit GlyRs expressed in a mammalian cell line (Takagi *et al.*, 1992). We therefore propose that, *in vivo*, gephyrin clusters the GlyR by interaction with the cytoskeleton and thereby facilitates cooperative receptor coupling. This may change the gating properties of the GlyR and possibly other neurotransmitter receptors upon assembly into a post-synaptic receptor matrix. Accordingly, the dense packing of receptors under the nerve terminal may be important not only for optimizing release geometry (Faber *et al.*, 1985), but also for improving neurotransmitter efficacy through cooperative receptor interactions.

Materials and methods

Nuclear injection

Oocytes from adult female *X.laevis* were treated with 2 mg/ml collagenase (type II, SIGMA) in calcium-free Barth solution for 2 h at room temperature. Defolliculated oocytes at stage V–VI (Methfessel *et al.*, 1986) were selected for nuclear injection (Voellmy and Rungger, 1982; Ballivet *et al.*, 1988) of a human α1 cDNA construct. The latter was generated by cloning a full-length cDNA encoding the human GlyR α1 subunit (Grenningloh *et al.*, 1990b) into the expression vector pCIS (Sontheimer *et al.*, 1989).

Recordings

Functional GlyR channels were analysed 2–6 days after cDNA injection. Agonist-activated currents were recorded using a conventional two-electrode voltage clamp set-up. The pipettes were filled with 1 M KCl solution and had resistances of ~3 and 0.5 MΩ, respectively, for the voltage probe and current injection. For high current levels, care was taken to minimize changes in chloride gradient and long lasting receptor desensitization. To establish dose–response curves, increasing concentrations of agonist (or antagonist) were applied, followed by long periods of washout (10–20 min). Maximal responses were repeatedly tested during the experiments, and cells displaying unstable currents were not considered.

The standard extracellular medium was frog Ringer solution (in mM: NaCl 115; KCl 1; $CaCl_2$ 1.8; HEPES 10; pH adjusted to 7.2 with NaOH).

Current analysis

The Hill equation (Hill, 1913) was used to describe monophasic dose–response distributions. For biphasic dose–responses, the two components were assumed to be independent, and a double sigmoid curve was fitted to the data points using the equation:

$$I = I_{max1} * \frac{A^{n1}}{A^{n1} + K_1{}^{n1}} + I_{max2} * \frac{A^{n}}{A^{n2} + K_2{}^{n2}}$$

where the parameters I_{max}, n and K represent maximal responses, Hill coefficients and EC_{50} (or IC_{50} for antagonists) values of the individual components and A, the ligand concentration. Parameters were estimated using a non-linear least-squares fitting method. The multiple correlation coefficient R^2 was used to test the quality of the fit and was generally >99.9%. For biphasic dose–responses, the Fisher test was used to determine whether the equation given above described the data adequately. In all cases examined, fits were highly significant ($P < 0.0001$).

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