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Glycine Receptor β Subunits Play a Critical Role in Potentiation of Glycine Responses by ICS-205,930

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ABSTRACT

The sensitivity of various types of recombinant glycine receptors (GlyRs) to ICS-205,930 was studied by fast perfusion in *Xenopus laevis* oocytes. This compound has previously been shown to potentiate glycine responses in rat spinal neurons between 10 nM and 1 μM, independently of its 5-HT_3 antagonist properties. In contrast, submicromolar concentrations of ICS-205,930 failed to affect responses of homomeric GlyRs formed from human α1 or α2 subunits, and micromolar concentrations (1–20 μM) acted differentially on the two types of homomeric receptors, potentiating the responses to glycine (10–20 μM) of α1 homomeric GlyRs and inhibiting the responses of α2 homomeric GlyRs. GlyRs β subunits markedly influenced the modulations induced by ICS-205,930. In oocytes expressing α1/β or α2/β heteromeric GlyRs, low concentrations of ICS-205,930 (20 nM–1 μM) induced a potentiation of glycine responses that was counteracted by an inhibitory effect at higher concentrations. Thus, GlyRs β subunits reduce by 2 orders of magnitude the concentration range potentiating α1-containing GlyRs and are required for potentiation of α2-containing GlyRs. These results reveal a new high-affinity potentiating site on GlyRs, to which β subunits participate. The difference in ICS sensitivity between α1 and α2 GlyRs cannot be explained by their difference in TM2 segment and extracellular domains partly conserved between glycine and 5-HT_3 receptors are probably involved in the interaction of some 5-HT_3 antagonists with GlyRs.

Previous electrophysiological data from cultured ventral spinal cord neurons (Chesnoy-Marchais, 1996) and from isolated purified motoneurons (Lévi et al., 1999) have shown that some 5-hydroxytryptamine (HT_3) antagonists potentiate the chloride response to Gly. Potentiations were detected for low concentrations (between 10 and 100 nM) of MDL-72222 (bemesetron) or ICS-205,930 (tropisetron) and occurred without changes in the leak current or reversal potential. Increasing the modulator concentration above 1 μM did not further enhance the potentiation but rather revealed an inhibitory effect. The glycinergic potentiating activity was not related to the 5-HT_3 antagonist activity of these compounds and resulted from a decrease of the EC_{50} value of glycine (Chesnoy-Marchais, 1996; Chesnoy-Marchais et al., 2000). The potentiating effect of ICS-205,930 (ICS) persisted in the presence of very high concentrations of Zn^{2+} (5–10 μM), ethanol (200 mM), or propofol (60–90 μM), indicating a new mechanism of action (Chesnoy-Marchais, 1999). It also persisted in excised patches (Chesnoy-Marchais, 1996), suggesting a direct interaction between ICS and Gly receptors (GlyRs).

Interestingly, binding data obtained from rat spinal cord membranes confirmed this idea (Maksay, 1998). The displacement of [^{3}H]strychnine binding by various 5-HT_3 ligands was compared in the absence and in the presence of 10 μM glycine. In the presence of glycine only, [^{3}H]strychnine binding could be displaced by submicromolar concentrations of the 5-HT_3 antagonists, which potentiate the electrophysiological response to glycine in this concentration range; on the contrary, the compounds known to reduce these responses (5-HT_3, *m*-chlorophenylbiguanide, *d*-tubocurarine; Chesnoy-Marchais, 1996) could displace [^{3}H]strychnine only at very high concentrations (Maksay, 1998). In addition, the displacement by Gly of [^{3}H]strychnine binding in the presence or absence of these substances showed that Gly affinity was slightly increased by potentiating agents, whereas it was decreased by inhibitory agents. These correlations between binding data and previous electrophysiological data have led Maksay (1998) to classify a series of chemicals as "Gly-positive", "Gly-negative", or "Gly-neutral" agents. This classification is coherent with all the electrophysiological data concerning modulations of GlyRs by 5-HT_3 ligands (Chesnoy-Marchais, 1996; Ren et al., 1999; Ye et al., 1999; Chesnoy-Marchais et al., 2000).

To further investigate the mechanisms of potentiation of Gly responses, we tested the effects of one of the 5-HT_3

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ABBREVIATIONS: HT, hydroxytryptamine; ICS, ICS-205,930; GlyR, glycine receptor; GABA, γ-aminobutyric acid; TM2, second transmembrane segment.

antagonists, ICS (a water-soluble compound), on different types of recombinant GlyRs expressed in *Xenopus laevis* oocytes. Inhibitory GlyRs belong to the superfamily of ligand-gated ionic channels including nicotinic receptors, $GABA_A$ receptors and 5-HT_3 receptors (Galzi and Changeux, 1994). They are pentamers that can be either homomeric (constituted of five identical α subunits) or heteromeric, including three α subunits and two β subunits—the latter being unable to form functional GlyRs in the absence of α subunits. In the spinal cord and brain stem, both $\alpha1$ and $\alpha2$ subunits can be expressed. Furthermore, it seems that the main GlyRs are homomeric $\alpha2$ GlyRs in the embryo, and heteromeric $(\alpha1)_3\beta_2$ GlyRs in the adult (Becker and Langosch, 1998). Up to now, the pharmacological agents able to clearly separate the different types of GlyRs are rare: the blocking agents picrotoxinin (one component of picrotoxin) and picrotoxin are more potent on homomeric GlyRs than on heteromeric GlyRs (Pribilla et al., 1992; Pistis et al., 1997), and another blocker, cyanotriphenylborate, has been reported to discriminate $\alpha1$ from $\alpha2$ homomeric GlyRs (Rundström et al., 1994). In the present study, we show that whereas the Gly responses of homomeric $\alpha2$ GlyRs are always inhibited by ICS (as already reported from an independent study; Maksay et al., 1999), the responses to low Gly concentrations of homomeric $\alpha1$ GlyRs can be potentiated by concentrations of ICS included between 1 and 40 μM. Furthermore, we show that association of β subunits with α subunits (either $\alpha1$ or $\alpha2$) strongly affects the sensitivity of GlyRs to ICS. The responses of both types of heteromeric GlyRs ($\alpha1/\beta$ and $\alpha2/\beta$) can be potentiated by submicromolar concentrations of ICS, which is never observed in the case of homomeric GlyRs. These results, briefly reported in an abstract (Chesnoy-Marchais and Supplisson, 1999), clearly demonstrate the presence of a high-affinity potentiating site on heteromeric GlyRs and indicate that GlyR β subunits contribute to this new modulatory site.

Materials and Methods

Heterologous Expression of GlyRs in *X. Laevis* Oocytes. Human GlyR $\alpha1$, $\alpha2$, $\alpha1$-G254A, and rat β subunit cDNAs (generous gifts from H. Betz's laboratory; Grenningloh et al., 1990a,b; Bormann et al., 1993) were transcribed in vitro using the appropriate (T7, T3, or SP6) mMessage-mMachine kits (Ambion, Austin, TX). cRNA (50 nl) were injected into *X. laevis* oocytes using a nanoliter injector (World Precision Instruments, Sarasota, FL) to reach a final amount of cRNA close to 50 ng in the case of homomeric GlyRs. For the expression of heteromeric GlyRs, the cRNA for the α and β subunits were mixed in a ratio of 1:4 and the final amount of injected cRNA coding for the α subunit was between 25 and 35 ng.

Defolliculated oocytes were isolated from *X. laevis* ovaries after 1 h of shaking incubation in a Ca^{2+}-free medium (84.5 mM NaCl, 5 mM HEPES, and 1 mM $MgCl_2$; pH adjusted to 7.6 with KOH) containing 1 mg/ml of collagenase type II (GIBCO, Grand Island, NY). Oocytes were kept at 19°C in individual wells containing 200 μl of Barth's medium (88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 10 mM HEPES, pH 7.6 adjusted with NaOH) with 50 μg/ml of gentamycin (GIBCO). Experiments were performed at room temperature (19–24°C), 3 to 5 days after cRNA injection.

Recording and Data Analysis. Using a two-electrode voltage-clamp and a OC-725C amplifier (Warner Instrument, Hamden, CT), currents were recorded in oocytes held at −70 mV. Oocytes were rapidly superfused using a closed chamber as described in (Supplisson and Bergman, 1997). All tubing was made of Teflon and changes in solution were achieved using a computer-controlled motorized valve (Omnifit, Cambridge, UK). The extracellular recording solution contained 100 mM NaCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, and its pH was adjusted to 7.4 with KOH. Repetitive applications of Gly in the absence of any modulator controlled the stability of the recording. The interval between successive Gly applications was chosen to allow complete recovery from desensitization, and was usually 40 s for Gly concentrations ≤50 μM and 90 or 120 s for higher Gly concentrations. When several Gly concentrations were tested on the same oocyte, the Gly concentration was increased progressively, to avoid possible irreversible desensitization of the receptors by the highest Gly concentrations. Experiments showing cumulative desensitization during repetitive applications were eliminated. The currents were filtered at 20 Hz and digitized at 100 Hz using a Digidata 1200A or 1320 and pClamp 6-8 software (Axon Instrument, Foster, CA). The leakage currents were subtracted on display. Glycine concentration response curves (Fig. 1) were fitted with Kaleidagraph (Abelbeck/Synergy Software, Reading, PA), using the equation:

$$R = R_{max}/[1 + (EC_{50}/[Gly])^{n}H] , \quad (1)$$

R is the amplitude of the response, and the parameters R_{max}, EC_{50}, and n_H are freely determined by the program. To clearly illustrate the bottom of these curves, the data and their fit are presented using logarithmic scales.

Drugs. A fresh stock solution of ICS (3-tropanyl-indole-3-carboxylate hydrochloride; RBI, Sigma) was prepared in distilled water at 1 mM and was kept on ice during the afternoon for the experiments using concentrations lower than 20 μM. For the experiments using higher concentrations of ICS, a 1 mM stock solution was prepared directly in the extracellular saline containing Gly. Picrotoxin (Sigma, St. Louis, MO) was diluted at 50 mM in ethanol. For testing the effect of picrotoxin, the same final concentration of ethanol (6/10,000) was present in all the solutions and did not affect the response. A given stock solution of Gly (Sigma), prepared at 100 mM in distilled water and kept at −20°C, was used for several months.

Results

Expression of Homomeric and Heteromeric GlyRs in *X. laevis* Oocytes: Glycine Sensitivity and Picrotoxin Sensitivity. To study modulations of responses to low concentrations of Gly, we expressed a high density of GlyRs (maximum response >10 μA). Such an expression would be expected to approach the situation encountered in native neurons, where the GlyRs are packed at high density in clusters (Béchade et al., 1996). Concentration-response curves obtained for homomeric GlyRs, constituted by either $\alpha1$ or $\alpha2$ human subunits, are illustrated in Fig. 1, A and B, respectively. A complete curve was recorded from a single oocyte (see under *Materials and Methods*) and fitted with a Hill equation (see figure legend for the values derived from each individual fit). The current traces illustrated in Fig. 1E (obtained from the oocyte expressing $\alpha1$ GlyRs) show that Gly concentrations higher than 100 μM induced some rapid desensitization during applications of only 4 s duration.

Similar experiments were performed in oocytes expressing heteromeric GlyRs, after injection of a mixture of cRNAs coding for the rat β subunit and the human $\alpha1$ or $\alpha2$ subunit (see under *Materials and Methods*). The efficacy of the method was checked by measuring the picrotoxin sensitivity of the response to 100 μM Gly. Table 1 gives the mean percentages of reduction induced by 10 μM and 30 μM picrotoxin, for oocytes injected only with α subunits ($\alpha1$ or $\alpha2$) cRNA, and for oocytes coinjected with β cRNA. The picrotoxin

sensitivity seen in oocytes coinjected with β cRNA was significantly lower from that of the corresponding homomeric α GlyRs ($P \leq .001$), indicating that heteromeric GlyRs were successfully expressed. Glycine responses recorded in the absence or presence of 10 μM picrotoxin from oocytes expressing homomeric or heteromeric GlyRs are illustrated in Fig. 1F. Glycine concentration-response curves obtained for heteromeric α1/β GlyRs and α2/β GlyRs are illustrated in Fig. 1, C and D, respectively. Table 2 gives the mean values of the Gly EC_{50} and the Hill coefficient, calculated for each type of GlyR by averaging the results obtained from the fit of each individual concentration-response curve.

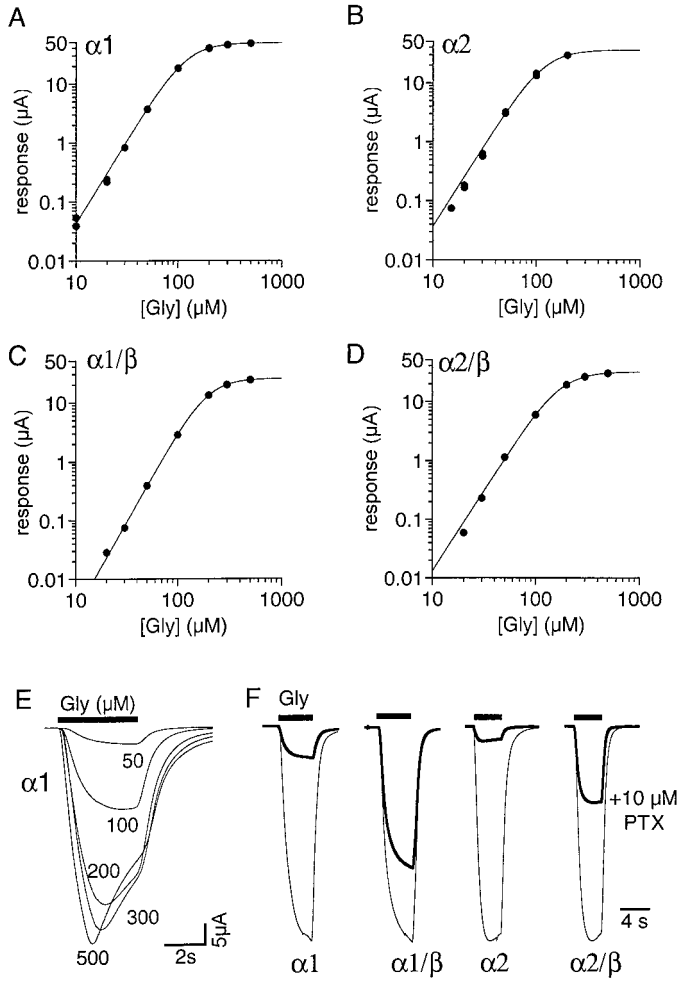

Fig. 1. Glycine sensitivity of recombinant GlyRs in *X. laevis* oocytes. A, concentration-response curve obtained from an oocyte expressing homomeric α1 GlyRs. The fit (continuous line) yields an EC_{50} = 120.9 ± 0.6 μM, n_H = 2.82 ± 0.03, and a maximum response of 49.26 ± 0.16 μA. B, concentration-response curve obtained from an oocyte expressing homomeric α2 GlyRs. The fit yields an EC_{50} = 119.4 ± 4.1 μM, n_H = 2.78 ± 0.14, and a maximum response of 37.1 ± 1.3 μA. C, concentration-response curve obtained from an oocyte expressing heteromeric α1/β GlyR. The fit yields an EC_{50} = 194.5 ± 0.8 μM, n_H = 3.07 ± 0.03, and a maximum response of 25.2 ± 0.1 μA. D, concentration-response curve obtained from an oocyte expressing heteromeric α2/β GlyRs. The fit yields an EC_{50} = 169.8 ± 0.6 μM, n_H = 2.74 ± 0.02, and a maximum response of 31.48 ± 0.09 μA. E, example of current traces recorded during successive applications of increasing concentrations of Gly on an oocyte expressing homomeric α1 GlyRs (same as in A). F, responses to 100 μM Gly recorded in the absence or in the presence of 10 μM picrotoxin (thick traces) from oocytes expressing homomeric α1 GlyRs (control response recorded in the absence of picrotoxin of 7.3 μA), heteromeric α1/β GlyRs (control response of 0.99 μA), homomeric α2 GlyRs (control response of 8.5 μA), or heteromeric α2/β GlyRs (control response of 5.4 μA; same oocyte as in D).

Modulation of Homomeric α1 GlyRs by ICS. To maximize potentiation of Gly responses by ICS, most experiments were performed using low concentrations of Gly (≤20 μM), as was the case in the previous evaluation of this modulation in spinal neurons (see also Fig. 3).

In the experiments performed here using human homomeric α1 GlyRs, no potentiation was ever detected using concentrations of ICS lower than 0.4 μM, in contrast to previous findings from rat neuronal receptors. ICS concentrations ranging from 1 to 40 μM did, however, potentiate the response of homomeric α1 GlyRs evoked by 10 to 20 μM Gly. In the experiment of Fig. 2A, as in most other experiments, ICS was applied only in the presence of Gly (15 μM). The potentiating effect of ICS was rapidly established, rapidly reversible, and increased with increasing concentrations of ICS up to 20 μM (Fig. 2, A, B, and D). When the ICS concentration was further increased (from 20 μM to 200 μM), the potentiation was progressively replaced by an inhibition, which was also rapidly established and rapidly reversible (Fig. 2C). Figure 2D gives the mean percentage of modulation of responses to 15 μM Gly as a function of the ICS concentration. The potentiation induced by a given concentration of ICS (10 μM) decreased with increasing concentrations of Gly and was no longer detectable when using 100 μM Gly (Fig. 3).

On a given oocyte, we compared the potentiations of Gly responses induced by brief applications of ICS (without preincubation, as in Fig. 2) with those induced by continuous applications of ICS (between and during successive Gly applications). The results (not shown) were not significantly different: the effective range of ICS concentrations was unchanged (ICS concentrations tested with both protocols on a same oocyte: 0.4, 1, and 10 μM) and ICS did not affect the current recorded in the absence of Gly.

Modulation of Homomeric α2 GlyRs. In the case of human homomeric α2 GlyRs, no potentiation of Gly re-

TABLE 1

Percentage of reduction of responses to 100 μM glycine induced by 10 and 30 μM picrotoxin in oocytes expressing different types of GlyRs

GlyR type	% Inhibition (mean ± S.D.)		*n*
	10 μM Picrotoxin	30 μM Picrotoxin	
α1	80.2 ± 3.5	91.5 ± 1.7	4
α1/β	41 ± 13	52 ± 14	7
α2	93.4 ± 0.4	97.6 ± 0.1	3
α2/β	68.3 ± 3.8	83.7 ± 2.0	3

TABLE 2

Mean values of the EC_{50} of glycine and Hill coefficient for each type of GlyR

A complete concentration-response curve was obtained from each oocyte and each curve was fitted by a Hill equation using kaleidagraph (see equation under *Materials and Methods*). Then, the EC_{50} and n_H derived from these fits were averaged for oocytes expressing the same type of GlyR. The range of maximum responses is also indicated.

GlyR Type	Gly EC_{50} (mean ± S.D.)	n_H (mean ± S.D.)	Range of Maximum Responses	*n*
	μM		μA	
α1	150 ± 45	2.8 ± 0.5	42–107	6
α1/β	210 ± 15	3.07 ± 0.04	20–25	3
α2	96 ± 28	3.2 ± 0.3	13–58	6
α2/β	157 ± 12	3.00 ± 0.37	31–74	3

sponses was observed, whatever the ICS or Gly concentrations tested (between 0.04 and 20 μM for ICS, between 7.5 and 50 μM for Gly). For example, we tested the effects of 0.04 to 1 μM ICS on the responses to low Gly concentrations (between 7.5 and 20 μM) in 16 different oocytes and observed either no modulation or, at 1 μM, a very small inhibitory effect (Fig. 4, A and D). Above 1 μM, ICS clearly reduced Gly responses in a concentration-dependent manner (Fig. 4, A, B, and D). This inhibitory effect was rapidly reversible (Fig. 4A) and was independent of the method used to apply ICS, without or with preincubation [compare Fig. 4B, middle traces, with Fig. 4C, left traces, from different oocytes; result confirmed by comparison of the effects induced without and with preincubation on a given oocyte (not shown)]. As illustrated by Fig. 4, C and E, the inhibitory effect of ICS increased slightly with increasing Gly concentrations between 10 and 50 μM.

Modulation of Heteromeric GlyRs. The modulation by ICS of the responses of heteromeric GlyRs to 20 μM Gly was studied for ICS concentrations ranging from 20 nM to 20 μM. Figure 5, A, B, and E, describes the results obtained from oocytes expressing α1 and β subunits, whereas Fig. 5, C, D, and F, describes the results obtained from oocytes expressing α2 and β subunits. Low concentrations of ICS, below 1 μM, potentiated the responses of both types of heteromeric GlyRs: for example, 20 nM ICS induced a potentiation of 20.3% $\pm$ 4.5% (n = 8) (mean $\pm$ S.E.M.; maximum 33.4%) in oocytes expressing α1 and β subunits and a potentiation of 12.2% $\pm$ 4.2% (n = 3) (mean $\pm$ S.E.M.; maximum 20.5%) in oocytes expressing α2 and β subunits. For both types of heteromeric GlyRs, the potentiation, which became more pronounced when the ICS concentration was increased, was maximal between 0.2 and 1 μM ICS (see Fig. 5, E and F, for the mean values of the percentage of potentiation; the maximum values of the percentage of potentiation induced by 0.2 and 1 μM ICS were 137% and 117.8%, respectively, for α1/β and 67.1% and 126.0%, respectively, for α2/β; see figure legend for the variability of the results). Increasing the ICS concentration above 1 μM revealed an opposite effect observed more easily in oocytes expressing α2 and β subunits than in oocytes expressing α1 and β subunits. In the experiment illustrated by Fig. 5, A and B (α1/β), potentiation was no longer detected when the concentration of ICS was increased to 20 μM, whereas a potentiation was transiently detected (Fig. 5A, arrow) during the washout of this high concentration (which can be understood because of the potentiating effect of submicromolar ICS concentrations on α1/β GlyRs). In the experiment illustrated by Fig. 5, C and D (α2/β), the percentage of potentiation was close to maximum already for 0.1 μM ICS,

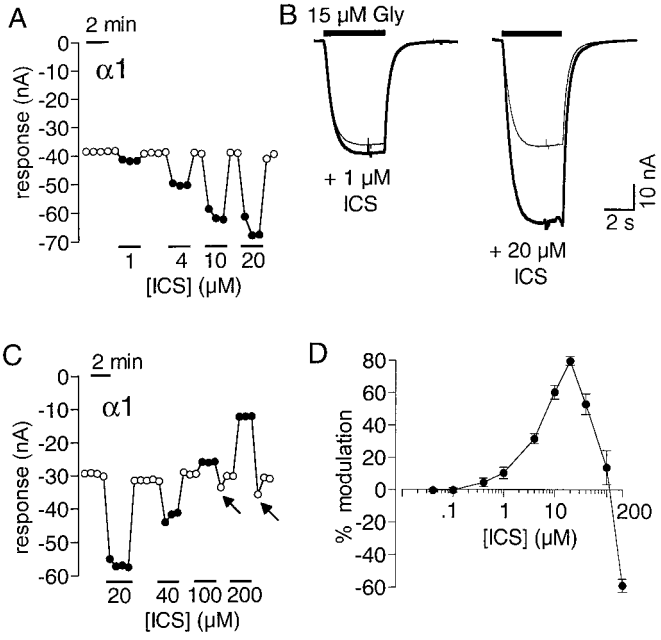

Fig. 2. Biphasic modulation of homomeric α1 GlyRs by ICS. A and B, data from a single oocyte repetitively exposed to 15 μM Gly. ICS was successively applied without preincubation at increasing concentrations, from 1 to 20 μM (as indicated by the bars in A). Each point in A gives the peak Gly response. In B, the current traces superimposed are means of two records obtained either in the presence of ICS (thick trace) or in the absence of ICS (one record before application, the other after wash, thin trace). C, data from another oocyte also repetitively exposed to 15 μM Gly. ICS was applied without preincubation at increasing concentrations, from 20 to 200 μM. Note the potentiation of the Gly response induced by 20 μM ICS (as in A) and the inhibition induced by 100 and 200 μM ICS. The transient rebounds (arrows) observed during washout of these high concentrations of ICS can be explained by the transient presence of ICS at a lower concentration, which potentiates the Gly response. D, mean results obtained from several experiments similar to those illustrated in A and B, using 15 μM Gly (n between 4 and 14, according to the ICS concentration). Below 0.4 μM, ICS does not significantly affect the responses; between 1 and 40 μM, ICS induces a potentiation, whereas above 100 μM, ICS induces an inhibition.

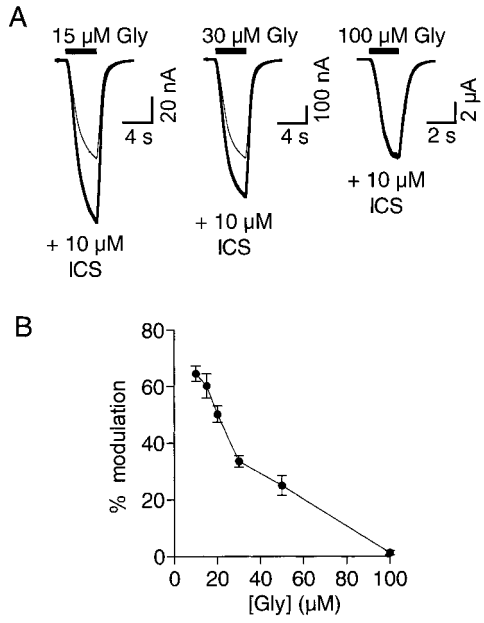

Fig. 3. Decrease of the ICS-induced potentiation of α1 GlyRs responses with increasing Gly concentrations. A, current traces recorded from a given oocyte, expressing homomeric α1 GlyRs, with or without 10 μM ICS, during applications of Gly at the concentrations indicated. The current scales (different for the three pairs of traces) are chosen to facilitate comparison of the effect of ICS for the different Gly concentrations (averages of two records and representation as in Fig. 2B). The effect of 10 μM ICS was tested several times on this oocyte, for each Gly concentration successively. To minimize the difficulties related to the desensitization induced by high Gly concentrations, the first test was performed for the lowest Gly concentration (repetitively applied without, with, and again without ICS), then the Gly concentration was increased and the next test was performed. The modulation induced by each ICS exposure was reversible and reproducible enough (during successive tests on the response to a same Gly concentration) to allow us to use this protocol. B, mean results obtained from several experiments similar to that illustrated in A (n between 3 and 11, according to the Gly concentration).

and decreased between 1 and 4 μM; a net inhibition of the Gly response was induced by 10 and 20 μM ICS, whereas a potentiation was observed during the washout of these high ICS concentrations (see arrows in Fig. 5C). Comparison of the mean results obtained from all the oocytes expressing heteromeric α1/β GlyRs (Fig. 5E) or α2/β GlyRs (Fig. 5F) with the results obtained from oocytes expressing the respective homomeric GlyRs clearly demonstrates the influence of β subunits on the modulation of Gly responses by ICS. For α1-containing GlyRs, β subunits markedly shift the ICS concentration range inducing potentiation toward lower values. For α2-containing GlyRs, β subunits allow potentiation by submicromolar concentrations of ICS, modulation never occurring in the absence of β subunits.

As already shown for homomeric α1 GlyRs (Fig. 3), the potentiation of Gly responses by a given concentration of ICS decreased with increasing Gly concentrations both in oocytes expressing heteromeric α1/β GlyRs (Fig. 5G) and in oocytes expressing heteromeric α2/β GlyRs (Fig. 5H).

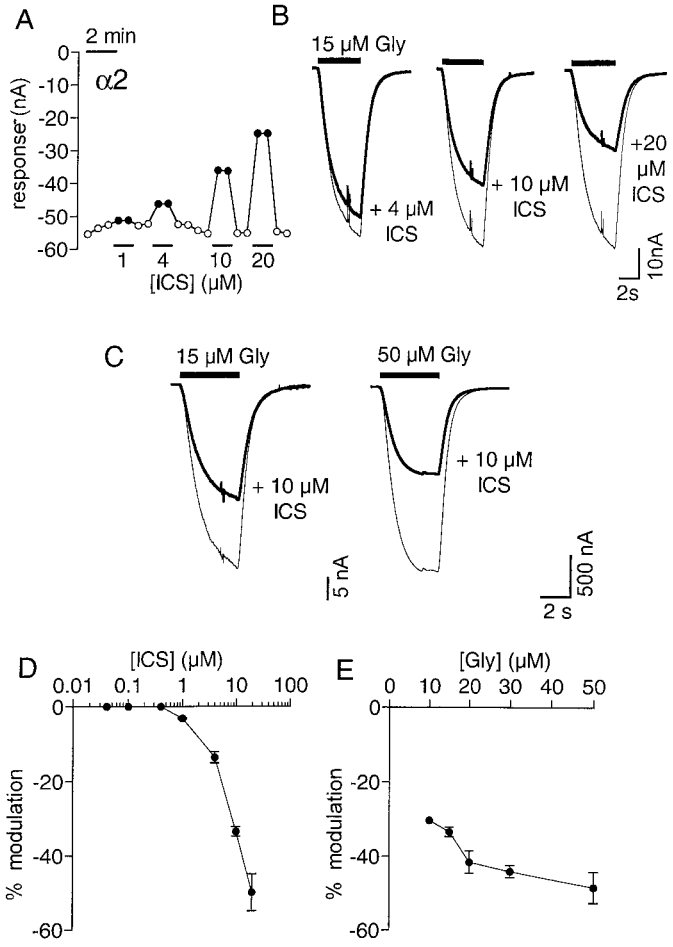

Fig. 4. ICS sensitivity of homomeric α2 GlyRs. A and B, data from a single oocyte repetitively exposed to 15 μM Gly. ICS was successively applied without preincubation at increasing concentrations (as indicated by the bars in A). Same representation as in Fig. 2, A and B. C, current traces obtained without or with 10 μM ICS (applied with preincubation) from an oocyte successively exposed in a repetitive way to different concentrations of Gly. Note the difference in current scale between the two pairs of traces (averages of 2 records and representation as in Fig. 2B). D, mean results obtained from several experiments similar to that illustrated in A, using 15 μM Gly (n between 3 and 9, according to the ICS concentration). Between 1 and 20 μM, ICS reduces these responses (A, B, and D), whereas at submicromolar concentrations, it has no effect (D). E, mean results obtained from 3 to 9 experiments similar to that illustrated in C.

The Difference in ICS Sensitivity between Homomeric α1 and α2 GlyRs Does Not Result from Their Difference in Transmembrane M2 Segments. In the case of GlyRs, as in the case of other ligand-gated channels of the same family, the second transmembrane segment (TM2) of each subunit, lining the ionic channel, is known to be involved in several types of modulations of the activity, in particular in the potentiation of Gly responses by alcohols and volatile anesthetics (see *Discussion*, below). Furthermore, the agents previously known to discriminate between different types of GlyRs, picrotoxinin and cyanotriphenylborate, were reported to act via TM2 segments (Pribilla et al., 1992; Rundström et al., 1994). The TM2 segments of α1 and α2 GlyR subunits differ only by one amino acid (the Gly-254 of α1, which is an alanine at the equivalent position in α2; Grenningloh et al., 1990b). We tested the effect of ICS on homomeric GlyRs obtained from the mutant subunit α1G254A (i.e., receptors having the same TM2 segments as homomeric α2 GlyRs). These GlyRs kept the ICS sensitivity of wild-type homomeric α1 GlyRs. Their response to 15 μM Gly was reversibly potentiated by supramicromolar concentrations of ICS (data not shown) and the mean percentages of potentiation [32.8% ± 2.4%, 66.1% ± 3.7%, and 74.2% ± 4.2% for 4, 10, and 20 μM ICS, respectively (mean ± S.E.M., n = 3)] were not significantly different from those obtained with homomeric wild-type α1 GlyRs (see Fig. 2).

Discussion

We have shown that recombinant GlyRs can be differentially modulated by ICS, depending on their subunit composition. Homomeric GlyRs formed from human α1 (Figs. 2 and 3) or α2 (Fig. 4) subunits were both insensitive to submicromolar ICS concentrations and were modulated in opposite directions by 1 to 20 μM ICS: α1 GlyRs were potentiated whereas α2 GlyRs were inhibited. The modulation of α1 GlyRs by ICS was biphasic, with an inhibition being induced above 100 μM ICS (Fig. 2, C and D; see also Maksay et al., 1999); this suggests the presence of two binding sites for ICS, the site of higher affinity accounting for the potentiation. In contrast with the situation encountered with homomeric GlyRs, in oocytes expressing heteromeric GlyRs, a potentiation of Gly responses was evoked by submicromolar ICS concentrations; in addition, a reverse modulation developed for higher ICS concentrations (Fig. 5). The difference in ICS sensitivity between homomeric and heteromeric GlyRs cannot be attributed to possible slight differences in Gly sensitivity (see Fig. 1 and references below), nor to differences in experimental conditions (very low Gly concentrations were used, especially for homomeric GlyRs, which might even have favoured their potentiation). Thus, ICS seems to be a useful tool to discriminate α1 from α2 homomeric GlyRs, and homomeric GlyRs from heteromeric GlyRs. In our experiments, the successful expression of heteromeric α1/β and α2/β GlyRs was demonstrated by their low picrotoxin sensitivity and high ICS sensitivity. The inhibitory effect of supramicromolar ICS concentrations might also seem weaker for oocytes expressing α1 and β rather than α2 and β (Fig. 5, E and F). However, this is likely to result from the simultaneous expression of homomeric GlyRs (differentially modu-

lated according to the α subunit type). This probably led to an underestimation of the potentiation of both types of heteromeric GlyRs by low ICS concentrations (which do not affect the fraction of the response carried by homomeric GlyRs). Note that difficulties in successfully expressing heteromeric GlyRs in *X. laevis* oocytes have already been encountered (Downie et al., 1996).

In the previous report concerning modulations by ICS of recombinant GlyRs in *X. laevis* oocytes (Maksay et al., 1999), only the inhibitory effect of high concentrations of ICS was observed; neither potentiation of homomeric α1 GlyRs by micromolar ICS concentrations nor potentiation of heteromeric GlyRs by submicromolar ICS concentrations were detected. Because the percentage of potentiation induced by a given ICS concentration clearly decreases with increasing Gly concentrations (Fig. 3; see also Fig. 7 in Chesnoy-Marchais, 1996), this discrepancy between the two studies can be explained by the difference in Gly concentrations used. We mainly used concentrations $\leq$20 μM, whereas Maksay et al. (1999) used half-saturating Gly concentrations that, in their experiments, were between 177 $\pm$ 19 μM and 807 $\pm$ 240 μM depending on the receptor type expressed. The pure inhibitory effect of ICS that we observed on homomeric α2 GlyRs was noncompetitive (Fig. 4E), in contrast to the previously reported inhibitory effect of atropine, another tropeine (Maksay et al., 1999).

Our results concerning heteromeric GlyRs agree qualitatively with previous results obtained from rat ventral spinal neurons (Chesnoy-Marchais, 1996) or purified motoneurons (Lévi et al., 1999). It was known that β subunits are involved in the interaction between GlyRs and gephyrin (Meyer et al., 1995), that they influence the elementary conductance states of the Gly-gated channels (Bormann et al., 1993) and lower their picrotoxinin sensitivity (Pribilla et al., 1992). However, all the amino acids affecting agonist binding to GlyRs belong to α subunits (Becker and Langosch, 1998), and the participation of β subunits to the gating process was never clearly demonstrated. There is no β subunit mutation that has been reported to affect GlyR function. The Gly EC_{50} is usually similar for homomeric and heteromeric GlyRs (Fig. 1; Pribilla et al., 1992; Takagi et al., 1992; Bormann et al., 1993; Rundström et al., 1994; Pistis et al., 1997). Furthermore, β subunits did not significantly affect the sensitivity of α1-containing GlyRs to various anesthetics (propofol, pentobarbitone, etomidate, and trichloroethanol; Pistis et al., 1997). By showing that coexpression of β subunits with α1 subunits lowers

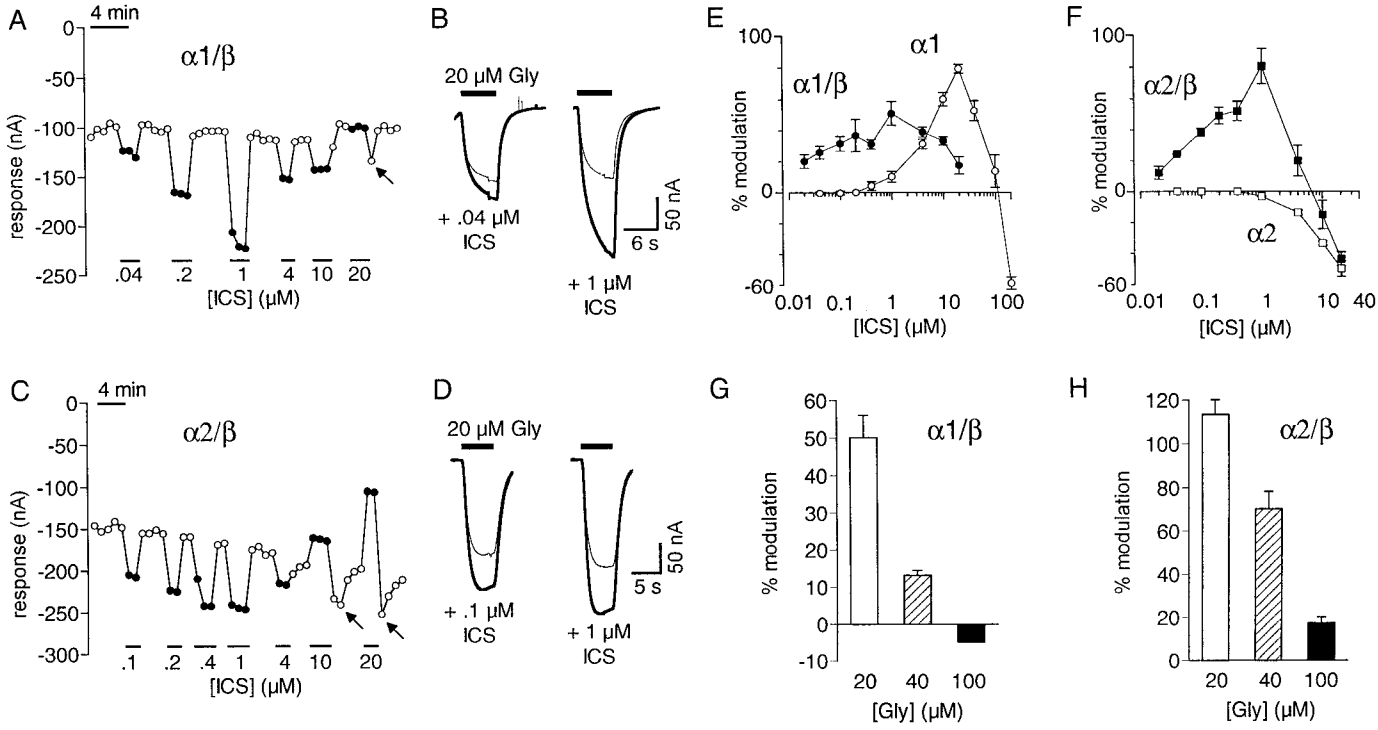

Fig. 5. Coexpression of GlyR β subunits with α1 or α2 subunits reveals potentiation of Gly responses by submicromolar concentrations of ICS. A and B, experiment performed on an oocyte expressing heteromeric α1/β GlyRs. Its response to 20 μM Gly was regularly measured, and ICS was successively applied without preincubation at increasing concentrations (as indicated by the bars in A). C and D, similar experiment performed on an oocyte expressing heteromeric α2/β GlyRs. Arrows in A and C indicate the rebounds observed just after wash of the highest concentrations of ICS tested (see text). E and F, mean results obtained from several oocytes coexpressing α1 and β subunits (●, n between 8 and 15 according to the ICS concentration) or α2 and β subunits (■, *n* between 3 and 8, according to the ICS concentration). To compare the results obtained for homomeric and heteromeric GlyRs [α1 (○) and α1/β (●) in E, α2 (□) and α2/β (■) in F], the data concerning homomeric receptors (already presented in Figs. 2D and 4D) have been redrawn. For oocytes expressing heteromeric GlyRs, note the significant potentiation already induced by low ICS concentrations ($\leq$ 0.2 μM). Note also the large error bars, indicating the strong variability of the results obtained from these oocytes; for example, in oocytes expressing α1 and β subunits, the minimum and maximum potentiations induced by 1 μM ICS were 16.4 and 117.8%, respectively. This variability is likely to originate from the simultaneous expression of heteromeric and homomeric GlyRs and from the variability of the fraction of heteromeric GlyRs expressed by each of these oocytes. G and H, percentage of modulation by 1 μM ICS of responses to different Gly concentrations (see *x* labels) recorded from oocytes expressing heteromeric α1/β GlyRs (G; $n = 5$) or heteromeric α2/β GlyRs (H; $n = 3$). Same protocol as in Fig. 3A (three successive tests of the effect of ICS on each oocyte, one for each Gly concentration). Note that G and H only include data from experiments using this protocol, whereas E and F include all the results obtained with the lowest Gly concentration. The potentiation induced by ICS was significantly lower for 40 μM Gly than for 20 μM Gly ($P \leq 0.01$). It was even smaller (H) or no longer detected and replaced by a small inhibition (G) for 100 μM Gly.

by about 2 orders of magnitude the threshold concentration of ICS required for potentiation of Gly responses, we demonstrate the involvement of β subunits in the transduction process between ligand binding and channel opening. Furthermore, the requirement of β subunits for potentiation by ICS of α2-containing GlyRs suggests that these subunits contribute to the high-affinity site recognizing this new class of glycinergic potentiator. The influence of β subunits on the sensitivity of GlyRs to ICS, contrasting with their lack of influence on the sensitivity to ethanol (Valenzuela et al., 1998) and various anesthetics (Pistis et al., 1997), confirms the difference between the mechanisms of potentiation involved (Chesnoy-Marchais, 1999).

The sequences of the human α GlyRs used here are almost identical in the extracellular domain to the corresponding rat α variants (Grenningloh et al., 1990b; Kuhse et al., 1991; Malosio et al., 1991). Thus, comparison of the present results with those obtained from rat embryonic spinal neurons (Chesnoy-Marchais, 1996; Lévi et al., 1999) confirms that these neurons express heteromeric GlyRs after 1 to 2 weeks in culture (Tapia and Aguayo, 1998; Lévi et al., 1999).

The potentiating effect of ICS on recombinant GlyRs reported here is significant but its magnitude is small (maximum a doubling of the response to 15–20 μM Gly); it is relatively smaller than in neurons, because the Gly concentrations used induced a smaller fraction of the maximum response in oocytes than in neurons. Quantitative differences in pharmacological properties between heterologous GlyRs in *X. laevis* oocytes and spinal GlyRs were already known. For example, the Gly EC_{50} are higher in oocytes (Fig. 1; Grenningloh et al., 1990b; Pistis et al., 1997; Mascia et al., 1998; Maksay et al., 1999) than in neurons ($EC_{50} \leq 40$ μM; Chesnoy-Marchais, 1996; Downie et al., 1996; Tapia and Aguayo, 1998; Lévi et al., 1999). The receptor density might partly account for this difference (Taleb and Betz, 1994). We expressed the receptors at a very high density and tried to superfuse the oocyte as rapidly and homogenously as possible. However, the size of the oocytes and their deeply folded membrane remain inappropriate to the study of responses to high, desensitizing, Gly concentrations. Other factors may also explain differences between results obtained from oocytes or neurons. For example, phosphorylation of GlyRs by protein kinase C controls their modulation by ethanol (Mascia et al., 1998) and the degree of modulation by ethanol of a given GlyR type depends on the expression system (Valenzuela et al., 1998). Differences in phosphorylation might account for differences between modulations observed in different cell types.

Identification of the amino acids involved in the potentiation of heteromeric GlyRs by low ICS concentrations could help in the design of more specific glycinergic potentiators. The modulations induced by other glycinergic potentiators, alcohols, and volatile anesthetics, are known to involve a serine conserved in TM2 segments of Gly and $GABA_A$ receptors (Mihic et al., 1997; see Krasowski and Harrison, 1999 for review of the role of TM2 segments in the potentiations induced by various anesthetics). This serine might participate in the binding site of these potentiators or influence transduction processes [it also affects agonists EC_{50} (Ueno et al., 1999) and modulations by molecules unlikely to all bind to the same site (Belelli et al., 1999)]. TM2 segments affect transduction processes for all the ligand-gated channels of the GlyRs superfamily [see Galzi et al. (1996) for nicotinic receptors TM2 mutants showing altered isomerization constants between inactive and active states without modification of binding constants; see Moorhouse et al. (1999) for an α1 GlyR TM2 mutant showing a decreased function and normal ligand binding; see also Boileau and Czajkowski (1999) for $GABA_A$ receptors TM2 mutations affecting modulation by benzodiazepines]. Up to now, the only agents known to discriminate between different GlyR types, picrotoxinin, and cyanotriphenylborate, recognize differences between TM2 segments of these receptors (Pribilla et al., 1992; Rundström et al., 1994). In contrast, we have shown that TM2 segments are not responsible for the striking difference in ICS sensitivity between homomeric α1 and α2 GlyRs (α1G254A GlyRs, having α2 TM2, showed the ICS sensitivity of wild-type α1 GlyRs). The binding site responsible for the potentiating effect of ICS on GlyRs (of much higher apparent affinity for heteromeric GlyRs than for α1 GlyRs, undetected for α2 GlyRs) is likely to involve some residues outside TM2, different on each subunit.

The binding of 5-HT_3 antagonists on 5-HT_3 receptors is known to involve a glutamate (E106 in mouse 5-HT_3 A_L receptors; Boess et al., 1997) and several tryptophan residues, probably involved in cation-Π interactions with the positive amine in the tropane of these antagonists (Venkataraman et al., 1999; Yan et al., 1999; Spier and Lummis, 2000). Interestingly, in the GlyR α1, α2, and β subunits, a glutamate is aligned with the E106 of 5-HT_3 A_L, and most residues aligned with the critical tryptophans are either tryptophan or phenylalanine, also able to participate in cation-Π interactions. Thus, the high-affinity binding site of ICS on GlyRs is likely to be extracellular and may resemble its site on 5-HT_3 receptors. Sequence comparisons and chimera analysis should facilitate identification of the residues responsible for the differential ICS sensitivity of the different GlyR types. Potentiation by ICS of α1 homomeric and heteromeric GlyRs could both be explained by binding of ICS at the interface between subunits, as described for benzodiazepines on $GABA_A$ receptors (Sigel and Buhr, 1997).

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