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Short communication

Functional characterisation of human glycine receptors in a fluorescence-based high throughput screening assay

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Abstract

The human glycine receptor subtypes α1β and α2 have been expressed stably in HEK293 cells, and the functional characteristics of the receptors have been characterised in the FLIPR® Membrane Potential Assay. The pharmacological properties obtained for nine standard ligands at the two receptors in this assay were found to be in good agreement with those from electrophysiology studies of the receptors expressed in *Xenopus* oocytes or mammalian cell lines. Hence, this high throughput screening assay will be of great use in future pharmacological studies of glycine receptors, particular in the search for novel compound structures acting at them.

Keywords: Glycine receptor; FLIPR® Membrane Potential Assay; High throughput screening

1. Introduction

The inhibitory neurotransmission mediated by glycine is important for a wide range of physiological functions throughout the CNS. Glycinergic synapses are especially abundant in the spinal cord, brain stem and caudal brain, where the amino acid contributes to the control of reflex responses, processing of sensory signals and motor rhythm generation via a family of strychnine-sensitive glycine receptors (Laube et al., 2002; Lynch, 2004; Rajendra et al., 1997). The glycine receptors belong to the superfamily of Cys-loop receptors, which also includes receptors for acetylcholine, γ-aminobutyric acid and serotonin. The glycine receptor is a ligand-gated ion channel complex composed of five subunits, which together form an extracellular domain possessing the orthosteric sites (the glycine binding sites) and an ion channel domain. Upon agonist binding to the orthosteric sites, the ion channel changes conformation and allows influx of anions like Cl^- and HCO_3^- into the cell (Keramidas et al., 2004; Laube et al., 2002; Lynch, 2004; Rajendra et al., 1997). To date five glycine receptor subunits have been cloned, termed α1–α4 and β, and homomeric α receptors as well as heteromeric α/β receptors have been shown to be functional in heterologous expression systems (Laube et al., 2002; Lynch, 2004; Rajendra et al., 1997). The homomeric and heteromeric glycine receptors are characterised by very similar pharmacological properties, the major differences being the larger single-channel conductances and the higher picrotoxin sensitivity exhibited by the homomeric receptor compared to the corresponding heteromeric receptor (Betz et al., 1999; Handford et al., 1996; Laube et al., 2002; Rajendra et al., 1997). The five glycine receptor subunits are differentially expressed in native tissues, and furthermore a developmental switch is observed when it comes to the identity of the major glycine receptor. Whereas the homomeric α2 receptor is the major prenatal glycine receptor subtype, the adult glycine receptors predominantly are heteromeric complexes composed of α1 and β subunits in a 2:3 stoichiometry (Grudzinska et al., 2005; Langosch et al., 1988; Takahashi et al., 1992).

In a recent study the pharmacological properties of a HEK293 cell line stably expressing the homomeric α1 glycine receptor were characterised in the fluorescence-

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based high throughput screening assay, the FLIPR® Membrane Potential assay (Jensen and Kristiansen, 2004). In the present study, HEK293 cell lines stably expressing the major physiological glycine receptor subtypes α1β and α2 have been constructed, and the functional properties of these receptors have been characterised.

2. Materials and methods

2.1. Materials

Culture media, serum, antibiotics and buffers for cell culture were obtained from Invitrogen (Paisley, Scotland). Glycine, taurine, β-alanine, (−)-strychnine (hereafter: strychnine), brucine, ω-[2′-phosphonomethyl[1,1′-biphenyl]-3-yl]alanine (PMBA), atropine and picrotoxin was purchased from Tocris Cookson. RU 5135 (3α-hydroxy-16-imino-5β-17-azaandrostan-11-one) was a kind gift from Dr. Mogens Nielsen (The Danish University of Pharmaceutical Sciences, Copenhagen, Denmark). The cDNAs for the human α1 and β glycine receptors subunits (hα1-pIRES-EGFP and hβ-pIRES-EGFP) were kind gifts from professor Peter R. Schofield (Garvan Institute of Medical Research, Sydney, New South Wales, Australia), and the cDNA encoding the human α2 glycine receptor (hα2-pCis) was a kind gift from professor Heinrich Betz (Max-Planck-Institute for Brain Research, Frankfurt, Germany).

2.2. Cell culture and generation of the stable cell lines

The glycine receptors were subcloned from their respective plasmids into pcDNA3.1/Hygro(−) (α1 and α2) or pcDNA3.1/Neo(+) (β) using *Eco*RI (in the case of α1 and β) or *Xba*I/*Not*I (in the case of α2) as restriction enzymes. Stable HEK293 cell lines expressing α1β and α2 glycine receptors were constructed essentially as described recently for a stable α1 glycine receptor-HEK293 cell line (Jensen and Kristiansen, 2004). Briefly, HEK293 cells were maintained at 37 °C in a humidified 5% CO_2 incubator in culture medium [Dulbecco's Modified Eagle Medium supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% dialyzed fetal bovine serum]. The cells were transfected with either hα1-pcDNA3.1/Hygro(−) and hβ-pcDNA3.1/Neo(+) or with hα2-pcDNA3.1/Hygro(−) using Polyfect as a DNA carrier according to the protocol by the manufacturer (Qiagen). The α1β-transfected cells were maintained for 2–3 weeks in culture medium containing 3 mg/ml G-418, 200 μg/ml hygromycin B and 10 μM strychnine, whereas the α2-transfected cells were cultured in medium containing 200 μg/ml hygromycin B and 10 μM strychnine. Antibiotic-resistant colonies were isolated, maintained in selection medium for 3–4 weeks and screened for functional responses to 3 mM glycine in the FLIPR® Membrane Potential assay (see below).

2.3. The FLIPR® membrane potential assay

The pharmacological properties of the α1β and α2 glycine receptor-HEK293 cell lines were characterised in the FLIPR® Membrane Potential assay (Molecular Devices). The cells were split into poly-D-lysine-coated black 96-well plates with clear bottom (BD Biosciences, Bedford, MA) in culture medium supplemented with the appropriate antibiotics and strychnine. 16–24 h later the medium was aspirated, and washed with 100 μL Krebs buffer [140 mM NaCl/4.7 mM KCl/2.5 mM $CaCl_2$/1.2 mM $MgCl_2$/11 mM HEPES/10 mM D-glucose, pH 7.4]. 50 μL Krebs buffer was added to each well (in the antagonist experiments, various concentrations of the antagonists were dissolved in the buffer). 50 μL of loading buffer (loading dye dissolved in Krebs buffer) was added to each well, and the plate was incubated at 37 °C in a humidified 5% CO_2 incubator for 30 min. The plate was assayed in a NOVOstar™ plate reader (BMG Labtechnologies) measuring emission [in fluorescence units (FU)] at 560 nm caused by excitation at 530 nm before and up to 1 min after addition of 25 μL agonist solution (agonist was dissolved in Krebs buffer). For the antagonist experiments, EC_{75}–EC_{90} concentrations of agonist were used (200 μM glycine and 500 μM glycine for the α1β and α2 glycine receptors, respectively). The experiments were performed in duplicate at least three times for each compound.

2.4. Data analysis

Concentration–response curves for agonists and antagonists were constructed based on the maximal responses at different concentrations of the respective ligands. The curves were generated by nonweighted least-squares fits using the program KaleidaGraph 3.6 (Synergy Software). Antagonist potencies were calculated from the inhibition curves using the "functional equivalent" of the Cheng–Prusoff equation $K_i = IC_{50}/[1+([A]/EC_{50})]$ (Craig, 1993), where $[A]$ is the agonist concentration used in the specific experiment.

3. Results and discussion

The antibiotic-resistant clones of the HEK293 cells lines transfected with the α1/β or α2 glycine receptor subunits were screened for functionality in the FLIPR® Membrane Potential assay using 3 mM glycine as the agonist concentration. Several functional clones were identified both for the α1β and the α2 glycine receptor cell line, and the two clones exhibiting the highest responses to maximal glycine stimulation were selected for further characterisation in the assay. Exposure of the selected α1β and α2 glycine receptor cell lines to glycine receptor agonists gave rise to solid increases in fluorescence intensities in the FLIPR® Membrane Potential assay, and these agonist responses could be suppressed in a concentration-dependent manner by

Table 1
Functional characteristics of the α1, α1β and α2 glycine receptor-HEK293 cell lines in the FLIPR® Membrane Potential Assay

	α1	α1β	α2
Agonists			
Glycine	82 (4.08±0.07)	89 (4.05±0.04)	290 (3.54±0.04)
	100	*100*	*100*
β-Alanine	180 (3.75±0.08)	420 (3.38±0.06)	580 (3.23±0.06)
	95±8	*92±11*	*78±12*
Taurine	490 (3.31±0.12)	1300 (2.89±0.05)	1600 (2.79±0.07)
	89±14	*82±9*	*45±9*
Antagonists			
Strychnine	0.11 (6.96±0.09)	0.14 (6.86±0.05)	0.32 (6.49±0.04)
Brucine	1.0 (5.99±0.06)	2.6 (5.59±0.04)	3.4 (5.46±0.05)
Picrotoxin	4.2 (5.38±0.13)	~ 100 (~ 4)	1.2 (5.92±0.06)
PMBA	3.5 (5.46±0.05)	6.8 (5.17±0.07)	6.7 (5.17±0.03)
RU 5135	0.021 (7.68±0.11)	0.042 (7.38±0.03)	0.033 (7.48±0.07)
Atropine	220 (3.66±0.11)	430 (3.36±0.06)	550 (3.26±0.06)

The EC_{50} values for the agonists are given in μM (with pEC_{50}±S.E.M in parentheses) and the R_{max} values for the agonists are given (as % of R_{max} of glycine) in italics below. The K_i or IC_{50} values of the antagonists are given in μM (with pK_i±S.E.M and pIC_{50}±S.E.M in parentheses). The data are the means of 3–10 individual experiments performed in duplicate. The data for the α1 glycine receptor-HEK293 cell line is from Jensen and Kristiansen (2004).

antagonists (data not shown). Both of the stable cell lines remained functional over multiple passages.

The α1β and α2 glycine receptor cell lines displayed very similar agonist pharmacologies in the FLIPR® Membrane Potential assay. For both cell lines the rank order of agonist potencies was glycine>β-alanine>taurine, the same rank order displayed by the α1 glycine receptor-HEK293 cell line (Table 1) (Jensen and Kristiansen, 2004). However, the three agonists appeared to be slightly more potent at the α1 glycine receptor than at the two other receptors, and furthermore taurine displayed a significantly lower efficacy at α2 than at α1 (Table 1 and Fig. 1A). Taurine has displayed varying efficacies at homomeric α1 glycine receptors in electrophysiology studies but it has not been reported to exhibit significantly different efficacies at α1 and α2 receptors (De Saint Jan et al., 2001; Lynch, 2004; Rajendra et al., 1997). The differences in potencies and efficacies displayed by the agonists at the glycine receptors could perhaps arise from different receptor expression levels in the three cell lines but this still remains to be investigated.

The rank orders of antagonist potencies at the α1 and α2 glycine receptors were RU 5135>strychnine>brucine≥PMBA≈picrotoxin>>atropine and RU 5135>strychnine>picrotoxin≥brucine≈PMBA>>atropine, respectively, and the K_i or IC_{50} values exhibited by the six antagonists did not differ significantly at the two homomeric receptors (Table 1). The similar pharmacological characteristics displayed by the two glycine receptors were not surprising considering the high amino acid sequence homology between the two, in particular in the regions constituting the orthosteric binding site targeted by several of the nine ligands in this study. The α1β glycine receptor displayed antagonist pharmacology similar to the two homomeric receptors, with the notable exception that picrotoxin was a significantly weaker antagonist at the heteromeric receptor (Table 1 and Fig. 1B). Thus, in concordance with the literature picrotoxin was found to be the only ligand shown capable of discriminating between homomeric and heteromeric glycine receptors (Handford et al., 1996; Lynch, 2004; Rajendra et al., 1997).

In conclusion, cell lines stably expressing the prototypic glycine receptor α1, the major fetal receptor α2 and the major adult receptor α1β have been constructed, and the pharmacological properties displayed by the receptors in the FLIPR® Membrane Potential assay are in good agreement with those obtained in electrophysiology studies of these ligands at the three recombinant receptors expressed in

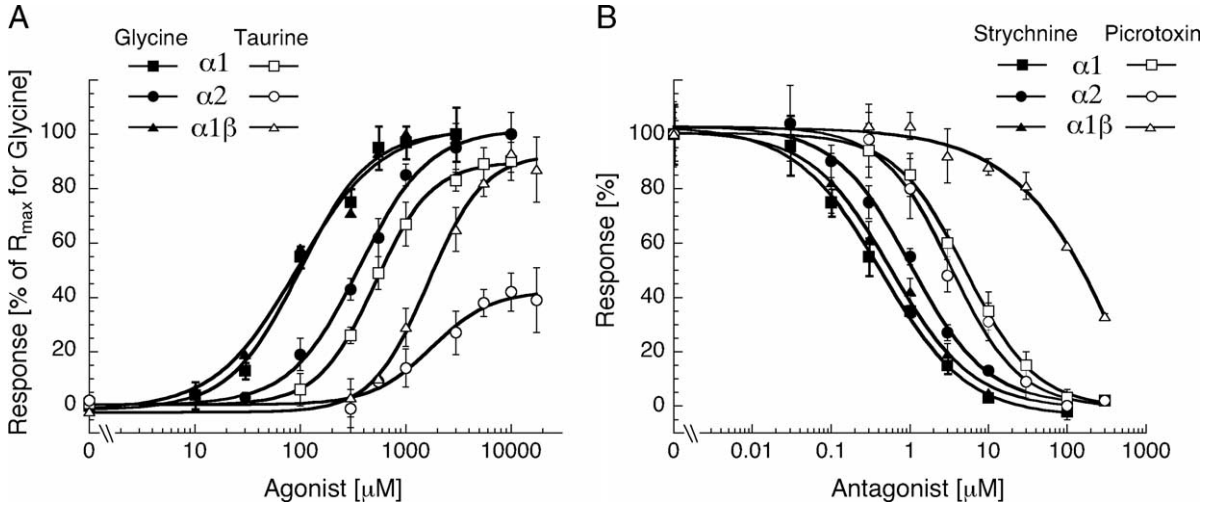

Fig. 1. Agonist and antagonist pharmacology of human glycine receptors in the FLIPR® Membrane Potential Assay. Concentration–response curves for agonists glycine and taurine (A) and antagonists strychnine and picrotoxin (B) at the α1, α1β and α2 glycine receptor-HEK293 cell lines. The agonist data are expressed as percentage of the maximal response of glycine at the respective receptor subtype, and the antagonist data as percentage of the response of 200, 200 and 500 μM glycine in the absence of antagonist at the α1, α1β and α2 receptors, respectively.

Xenopus oocytes or in mammalian cell lines (De Saint Jan et al., 2001; Handford et al., 1996; Laube et al., 2002; Lynch, 2004; Maksay et al., 1999; Rajendra et al., 1997; Saitoh et al., 1994). Although the set-up of the assay does not allow for sophisticated and detailed studies of GlyR signalling kinetics, the assay will still be useful in future studies of the glycine receptors. Furthermore, the high reproducibility and the high throughput of the fluorescence-based assay could enable the screening of libraries of structurally diverse compounds at the three receptors. Novel structures identified in such screenings could potentially possess more pronounced selectivities for specific glycine receptor subtypes than the ligands currently available.

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References

Betz, H., Kuhse, J., Schmieden, V., Laube, B., Kirsch, J., Harvey, R.J., 1999. Structure and functions of inhibitory and excitatory glycine receptors. Ann. N. Y. Acad. Sci. 868, 667–676.

Craig, D.A., 1993. The Cheng–Prusoff relationship: something lost in the translation. Trends Pharmacol. Sci. 14, 89–91.

De Saint Jan, D., David-Watine, B., Korn, H., Bregestovski, P., 2001. Activation of human α1 and α2 homomeric glycine receptors by taurine and GABA. J. Physiol. 535, 741–755.

Grudzinska, J., Schemm, R., Haeger, S., Nicke, A., Schmalzing, G., Betz, H., Laube, B., 2005. The beta subunit determines the ligand binding properties of synaptic glycine receptors. Neuron 45, 727–739.

Handford, C.A., Lynch, J.W., Baker, E., Webb, G.C., Ford, J.H., Sutherland, G.R., Schofield, P.R., 1996. The human glycine receptor beta subunit: primary structure, functional characterisation and chromosomal localisation of the human and murine genes. Brain Res. Mol. Brain Res. 35, 211–219.

Jensen, A.A., Kristiansen, U., 2004. Functional characterisation of the human a1 glycine receptor in a fluorescence-based membrane-potential assay. Biochem. Pharmacol. 67, 1789–1799.

Keramidas, A., Moorhouse, A.J., Schofield, P.R., Barry, P.H., 2004. Ligand-gated ion channels: mechanisms underlying ion selectivity. Prog. Biophys. Mol. Biol. 86, 161–204.

Langosch, D., Thomas, L., Betz, H., 1988. Conserved quaternary structure of ligand-gated ion channels: the postsynaptic glycine receptor is a pentamer. Proc. Natl. Acad. Sci. U. S. A. 85, 7394–7398.

Laube, B., Maksay, G., Schemm, R., Betz, H., 2002. Modulation of glycine receptor function: a novel approach for therapeutic intervention at inhibitory synapses? Trends Pharmacol. Sci. 23, 519–527.

Lynch, J.W., 2004. Molecular structure and function of the glycine receptor chloride channel. Physiol. Rev. 84, 1051–1095.

Maksay, G., Laube, B., Betz, H., 1999. Selective blocking effects of tropisetron and atropine on recombinant glycine receptors. J. Neurochem. 73, 802–806.

Rajendra, S., Lynch, J.W., Schofield, P.R., 1997. The glycine receptor. Pharmacol. Ther. 73, 121–146.

Saitoh, T., Ishida, M., Maruyama, M., Shinozaki, H., 1994. A novel antagonist, phenylbenzene omega-phosphono-alpha-amino acid, for strychnine-sensitive glycine receptors in the rat spinal cord. Br. J. Pharmacol. 113, 165–170.

Takahashi, T., Momiyama, A., Hirai, K., Hishinuma, F., Akagi, H., 1992. Functional correlation of fetal and adult forms of glycine receptors with developmental changes in inhibitory synaptic receptor channels. Neuron 9, 1155–1161.