Neuroscience 146 (2007) 427–434

GLYCINE RECEPTORS ARE FUNCTIONALLY EXPRESSED ON BULLFROG RETINAL CONE PHOTORECEPTORS

L.-H. GE,[a1] S.-C. LEE,[a1] J. LIU[a,b] AND X.-L. YANG[a]*

[a]*Institute of Neurobiology, Institutes of Brain Science and State Key Laboratory of Medical Neurobiology, Fudan University, 138 Yixueyuan Road, Shanghai 200032, China*

[b]*Institutes for Biological Sciences, Chinese Academy of Sciences, Graduate School of the Chinese Academy of Sciences, Shanghai 200031, China*

Abstract—Using immunocytochemical and whole cell recording techniques, we examined expression of glycine receptors on bullfrog retinal cone photoreceptors. Immunofluorescence double labeling experiments conducted on retinal sections and isolated cell preparations showed that terminals and inner segments of cones were immunoreactive to both α1 and β subunits of glycine receptors. Moreover, application of glycine induced a sustained inward current from isolated cones, which increased in amplitude in a dose-dependent manner, with an EC_{50} (concentration of glycine producing half-maximal response) of 67.3±4.9 μM, and the current was blocked by the glycine receptor antagonist strychnine, but not 5,7-dichlorokynurenic acid (DCKA) of 200 μM, a blocker of the glycine recognition site at the *N*-methyl-D-aspartate (NMDA) receptor. The glycine-induced current reversed in polarity at a potential close to the calculated chloride equilibrium potential, and the reversal potential was changed as a function of the extracellular chloride concentration. These results suggest that strychnine-sensitive glycine receptors are functionally expressed in bullfrog cones, which may mediate signal feedback from glycinergic interplexiform cells to cones in the outer retina.

Key words: glycine receptor, whole cell recording, cone photoreceptor, retina, immunofluorescence double labeling.

Glycine, in addition to GABA, works as a major inhibitory neurotransmitter in the retina, and plays an important physiological role by activating the glycine receptor (GlyR). GlyR is a ligand-gated chloride channel, which is composed of four ligand binding α-subunits (α1, α2, α3 and α4) and a structural β-subunit (Lynch, 2004). In the amphibian retina, there are glycinergic interplexiform cells and amacrine cells (Smiley and Basinger, 1988; Smiley and Yazulla, 1990; Wu and Maple, 1998; Deng et al., 2001). Whereas the former send processes to the outer retina, thus providing an intraretinal feedback pathway (Smiley and Basinger, 1988; Smiley and Yazulla, 1990), the latter mainly provide input to terminals of bipolar cells through reciprocal synapses between bipolar cells and amacrine cells (Maple and Wu, 1998; Wu and Maple, 1998). Like in mammalian retinas (Grünert and Wässle, 1993; Wässle et al., 1998; Lin et al., 2000; Haverkamp et al., 2003, 2004), GlyRs are extensively distributed across the inner retina in lower vertebrates (Smiley and Yazulla, 1990; Yazulla and Studholme, 1991, 2001; Zucker and Ehinger, 1993; Vitanova et al., 2004; Vitanova, 2006). In agreement with the immunocytochemical data, glycine-elicited currents have been recorded in various retinal neurons, including bipolar, amacrine and ganglion cells (Han et al., 1997; Solessio et al., 2002; Du and Yang, 2002a,b; Li and Yang, 2003). For instance, in the bullfrog retina, glycine-induced currents were elicited at both dendrites and axon terminals of bipolar cells, which were supposed to be driven by glycinergic interplexiform cells and amacrine cells respectively (Du and Yang, 2002a,b). Immunofluorescence labeling for GlyRs was also found in the outer retina of both mammals and non-mammals (Smiley and Yazulla, 1990; Yazulla and Studholme, 1991; Grünert and Wässle, 1993; Sassoè-Pognetto et al., 1994; Vitanova et al., 2004; Lee et al., 2005; Vitanova, 2006). In bullfrog, labeling for both GlyR α1 and GlyR β is seen in the outer plexiform layer (OPL) in addition to Müller cells (Lee et al., 2005). The elements with this labeling are commonly thought to be processes of cells postsynaptic to photoreceptors, such as bipolar cells and horizontal cells. An immunocytochemical investigation previously showed the presence of GlyR-immunoreactivity in the cytoplasm of the connecting axon of a subset of cone photoreceptors in the goldfish retina, but not in the cone plasma membrane (Yazulla and Studholme, 1991). In the macaque monkey retina, single GlyR-labeled dots are visible in some cone inner segments (Grünert and Wässle, 1993). Most recently, immunofluorescence labeling for GlyRs was found in a minority of porcine cone photoreceptors and expression of the GlyR β subunit was demonstrated in a similar population of cones by RT-PCR. Indeed, glycine application could induce large currents from these cells (Balse et al., 2006). In the present work, functional expression of GlyRs was studied in the bullfrog retina using double immunofluorescence labeling and whole cell recording techniques. Our results demonstrate that labeling for the GlyR α1 and β subunits is found in bullfrog cone photoreceptors and glycine application in-

[1] The first two authors contributed equally to this work.

*Corresponding author. Tel: +1-8621-6422-1975; fax: +1-8621-5423-7647.

E-mail address: xlyang@fudan.edu.cn (X.-L. Yang).

Abbreviations: $[Cl^-]_o$, extracellular chloride concentration; DCKA, 5,7-dichlorokynurenic acid; DMSO, dimethyl sulfoxide; E_{Cl}, chloride equilibrium; EC_{50}, concentration of glycine producing half-maximal response; E_{rev}, reversal potential; GlyR, glycine receptor; INL, inner nuclear layer; IPL, inner plexiform layer; MW, molecular weight; ONL, outer nuclear layer; OPL, outer plexiform layer; PB, phosphate buffer.

0306-4522/07$30.00+0.00 © 2007 IBRO. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.neuroscience.2007.01.033

duces currents in a majority of the cones through activation of strychnine-sensitive GlyRs.

EXPERIMENTAL PROCEDURES

Immunocytochemistry and confocal microscopy

Preparations for immunolabeling were made from adult bullfrog (*Rana catesbeiana*), as previously described in detail (Zhao and Yang, 2001). Animal treatments were in accordance with the NIH guidelines for animal experimentation and the guidelines of Fudan University on the ethical use of animals. Adequate care was taken to minimize the number of animals used and their suffering. Briefly, the posterior eyecup was immersion-fixed in fresh 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4) for 10 min at 4 °C. After fixation, the retinas were sequentially cryoprotected in 10% (w/v), 20% and 30% sucrose in 0.1 M PB and then embedded in OCT (Miles Inc., Elkhart, IN, USA). They were cut vertically by a freezing microtome (Leica, Nussloch, Germany) at 14 μm thickness, and the sections were preincubated in 0.1 M PBS containing 6% normal donkey serum and 0.2% Triton X-100. Goat polyclonal antibodies against GlyR α1 (C-15, 1:250), β (N-20, 1:250) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used as the primary antibodies. For immunofluorescence double-labeling, one of the primary antibodies was used together with the rabbit anti-recoverin polyclonal antibody (1:1000, Chemicon, Temecula, CA, USA), for labeling photoreceptors (McGinnis et al., 1997). The sections were then incubated in DT-PBS containing a mixture of two secondary antibodies: FITC-conjugated donkey anti-rabbit IgG (1:200, Jackson Immunoresearch Laboratories, West Grove, PA, USA) for recoverin; donkey anti-goat IgG tagged with Texas Red (1:200, Jackson Immunoresearch Laboratories) for α1 and β. Omission of one primary antibody yielded only the immunoreactivity for the remaining antibody and omission of both abolished any immunolabeling. Staining by a mixture of the two secondary antibodies after incubating with one of the two primary antibodies showed no cross-reactivity of species-specific secondary antibodies. Immunostaining vanished when the α1 and β antibodies were pre-absorbed with the immunizing antigens (sc-17278 P for α1, sc-17283 P for β, Santa Cruz Biotechnology).

Immunohistochemical experiments were also performed in isolated cones, following the procedure described in detail previously (Yu et al., 2006), with minor modifications. Isolated cones were obtained following the procedure used for preparing isolated cells for patch-clamp recording (see below). The cells were fixed and then incubated with the primary antibodies for 2 h and further incubated with the secondary antibodies for 30 min at room temperature.

Fluorescently labeled sections/cells were visualized with a Leica SP2-AOBS confocal laser-scanning microscope (Leica, Mannheim, Germany), using a 63× oil-immersion objective lens. Single optical sections were made through the preparation at intervals of 1.0 μm. Differential interference contrast (DIC) images of the identical scope were also taken for comparison.

Western blot analysis

Bullfrog retina extracts were prepared following the procedure described in detail by Zhao and Yang (2001), with minor modifications. The extract samples (2.0 mg/ml, 20 μl) were loaded, subjected to 12% SDS-PAGE and electroblotted onto polyvinylidene fluoride (PVDF) membranes using Mini-PROTEAN 3 Electrophoresis System and Mini Trans-Blot Electrophoretic Transfer System (Bio-Rad, Hercules, CA, USA). The membranes were blocked with non-fat milk at room temperature for 2 h, and then incubated with the antibody against the α1 or β, at a working dilution of 1:500, overnight at 4 °C. The blots were washed, incubated with horseradish peroxidase–conjugated donkey anti-goat IgG (1:5000; Santa Cruz Biotechnology) for 2 h at 4 °C, and finally visualized with enhanced chemiluminescence (Amersham Biosciences, USA).

Whole cell patch clamp recordings

Retinal neurons were dissociated by enzymatic and mechanical treatment, described previously in detail (Lu et al., 1998), with minor modifications. Retinal pieces were first incubated in Ringer's with 75 U/ml papain, 1 mg/ml cysteine for 30 min at 30 °C. Ringer's solution contained (in mM) 120 NaCl, 2 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 Hepes and 10 glucose, with Phenol Red (0.001% v/v), adjusted to pH 7.4 with NaOH. Cells were freshly isolated by mechanical trituration, cone photoreceptors can be easily identified by their characteristic morphological features (see "Results").

Whole-cell membrane currents of cones, voltage-clamped at −60 mV, were recorded with pipettes of 5~6 MΩ resistance when filled with a solution containing (in mM) 128 CsCl, 2 $MgCl_2$, 1 $CaCl_2$, 10 EGTA, 10 Hepes (pH adjusted to 7.4 with CsOH). Liquid junction potential of the pipettes was calculated and auto-compensated by the software Pulse 8.52 (HEKA). Fast capacitance current caused by the electrode was always fully cancelled and cell capacitance was partially cancelled by the circuit of the amplifier. Eighty percent of the series resistance of the recording electrodes was compensated. Data were acquired at a rate of 10 kHz, filtered at 2 kHz and then stored for further off-line analysis. Dose-response relationships of glycine-induced currents were fitted to the Hill equation: $I/I_{max}=1/(1+(EC_{50}/[glycine])^n)$, where I is the current response elicited by a given glycine concentration [glycine], I_{max} is the response at a saturating concentration of glycine, EC_{50} is the concentration of glycine producing half-maximal response, and n is the Hill coefficient. Data are presented as mean±S.E.M.

Solutions and drug application

5,7-Dichlorokynurenic acid (DCKA) was first dissolved in dimethyl sulfoxide (DMSO) as stock solution, and the final concentration of DMSO in Ringer's was minimized, showing no effects on glycine-induced currents from bullfrog cones. Other drugs were dissolved in Ringer's. Solutions were delivered using a stepper motor-based rapid solution changer RSC-100 (Biological Science Instruments, Claix, France), as described in detail previously (Lu et al., 1998). The recorded cells were lifted from the bottom of the dish, and completely bathed in the solution. The solution exchange was completed in 20 ms in this work. All chemicals were purchased from Sigma (St. Louis, MO, USA), unless otherwise specified.

RESULTS

Expression of GlyR α1 and GlyR β subunits in bullfrog cones

The specificity of the antibodies used in this study was initially assessed by Western blotting. Western blot analysis with GlyR α1 revealed a single band at approximately 100 kDa (Fig. 1a), almost doubling the molecular weight (MW) of GlyR α1 (48 kDa) of rat spinal cord (Langosch et al., 1988). The single band revealed with GlyR β was around 112 kDa (Fig. 1b), doubling the MW of rat GlyR β (56 kDa) (Langosch et al., 1988). These bands appeared to represent the dimeric forms of GlyR α1 and β respectively. No band was detectable when GlyR α1 (Fig. 1a′) and GlyR β (Fig. 1b′) were respectively preabsorbed with the corresponding immunizing antigens. The specificity of the immunofluorescence labeling was further evaluated with immunizing antigens, as shown in Fig. 1A_4 and B_4. Only weak labeling in the inner segments of photorecep-