Brain Research Bulletin 100 (2014) 1–5

Contents lists available at ScienceDirect

Brain Research Bulletin

journal homepage: www.elsevier.com/locate/brainresbull

Research report

Contaminating levels of zinc found in commonly-used labware and buffers affect glycine receptor currents

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ARTICLE INFO

Article history:
Received 4 September 2013
Received in revised form 18 October 2013
Accepted 21 October 2013
Available online 28 October 2013

Keywords:
Zinc
Tricine
Allosteric modulation
Glycine receptor
Electrophysiology
Xenopus oocytes

ABSTRACT

Zinc is an allosteric modulator of glycine receptor function, enhancing the effects of glycine at nM to low μM concentrations, and inhibiting its effects at higher concentrations. Because of zinc's high potency at the glycine receptor, there exists a possibility that effects attributed solely to exogenously-applied glycine in fact contain an undetected contribution of zinc acting as an allosteric modulator. We found that glycine solutions made up in standard buffers and using deionized distilled water produced effects that could be decreased by the zinc chelator tricine. This phenomenon was observed in three different vials tested and persisted even if vials were extensively washed, suggesting the zinc was probably present in the buffer constituents. In addition, polystyrene, but not glass, pipets bore a contaminant that enhanced glycine receptor function and that could also be antagonized by tricine. Our findings suggest that without checking for this effect using a chelator such as tricine, one cannot assume that responses elicited by glycine applied alone are not necessarily also partially due to some level of allosteric modulation by zinc.

1. Introduction

The glycine receptor (GlyR) is a member of the cys-loop superfamily of ligand-gated ion channels. GlyR are pentameric in structure and can be either composed solely of α subunits (homomeric), or heteromerically in the form of α & β subunits arranged around a central chloride-conducting pore. To date, three α and one β subunits have been described in humans (Baer et al., 2009). Responsible for the majority of fast inhibitory neurotransmission in the brainstem and spinal cord, GlyR are also found in many higher brain regions including the hippocampus, nucleus accumbens, and prefrontal cortex (Baer et al., 2009; Jonsson et al., 2012, 2009; Lynch, 2004). GlyR function is modulated by inhaled anesthetic, alcohols and inhalants, making it a possible target for the development of therapeutics for the treatment of alcoholism (Beckstead et al., 2000; Mascia et al., 1996; Tipps et al., 2010).

Zinc is a biphasic allosteric modulator of GlyR function, enhancing GlyR currents at concentrations below 10 μM while inhibiting receptor function at higher concentrations (Harvey et al., 1999; Laube et al., 2000, 1995). Recent studies have shown that zinc is a contaminant present at low nM concentrations in physiological solutions and in commonly-used labware (Kay, 2004; McCracken et al., 2010). Due to an increasing concern that some of the results of our studies may have been due to an effect of glycine in conjunction with zinc, rather than glycine itself, we utilized an electrophysiological approach to examine the effects of background contaminating zinc levels and to distinguish among several possible sources of zinc contamination in reagents and labware used in the preparation of solutions for electrophysiological recordings.

2. Materials and methods

2.1. Reagents

Except NaOH [Cat. No. SS255] obtained from Fisher (Pittsburgh, PA), all other chemicals were purchased from Sigma-Aldrich (St Louis, MO) including NaCl [Cat. No. S9625], $CaCl_2$ [C7902], KCl [P5405], $MgSO_4$ $7H_2O$ [2030391], $Ca(NO_3)_2$ $4H_2O$ [C1396], HEPES [H3375], $NaHCO_3$ [S3817], tricine [T5816] and glycine [G7126]. Distilled water was purified further using a Barnstead E-Pure Ultrapure D4641 Water Purification System.

2.2. Oocyte isolation and cDNA injection

Xenopus laevis were obtained from Nasco (Fort Atkinson, WI) and housed at room temperature on a 12-h light/dark cycle. Oocytes were obtained via surgery, performed in accordance with AAALAC

Abbreviations: EC5, effective concentration producing 5% of maximal effect; GlyR, glycine receptor; ICP-MS, inductively-coupled plasma mass spectrometer; MBS, modified Barth's saline; *N,N,N′,N′*-tetrakis(2-pyridylmethyl)ethane-1,2-diamine, TPEN; Tri, tricine.

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http://dx.doi.org/10.1016/j.brainresbull.2013.10.012

regulations, and placed in isolation media containing 108 mM NaCl, 1 mM EDTA, 2 mM KCl, and 10 mM HEPES. Forceps were used to manually remove the thecal and epithelial layers from stage V and VI oocytes followed by removal of the follicular layer using a 10-min incubation in 0.5 mg/mL Sigma type 1A collagenase in buffer containing 83 mM NaCl, 2 mM $MgCl_2$, and 5 mM HEPES. Oocytes were injected through their animal poles with 30 nL of α1β glycine receptor subunit cDNA (at a 1:20 α1:β ratio) in a modified pBK-cytomegalovirus vector (Mihic et al., 1997), using a micropipette (10–15 μm tip size) attached to an electronically-activated microdispenser. Oocytes were stored in the dark at room temperature for 24 h followed by subsequent storage in the dark at 19 °C for up to 5 days post-injection in 96-well plates containing modified Barth's saline (MBS) [88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 10 mM HEPES, 0.82 mM $MgSO_4{\cdot}7H_2O$, 0.33 mM $Ca(NO_3)_2$, 0.91 mM $CaCl_2$ at pH 7.5] supplemented with 2 mM sodium pyruvate, 0.5 mM theophylline, 10 U/mL penicillin, 10 mg/L streptomycin and 50 mg/L gentamicin, and sterilized by passage through a 0.22 μm filter.

2.3. Two-electrode voltage-clamp electrophysiology

Oocytes expressed heteromeric GlyR within 48 h, and all electrophysiological recordings were made within 5 days of cDNA injection. Oocytes were placed in a 100 μL bath with the animal poles facing upwards and impaled with two high-resistance (0.5–10 MΩ) glass electrodes filled with 3 M KCl. Cells were voltage-clamped at −70 mV using an OC-725 C oocyte clamp (Warner Instruments, Hamden, CT) and perfused with MBS at a rate of 2 mL/min using a Masterflex USA peristaltic pump (Cole Parmer Instrument Co., Vernon Hills, IL) through 18-gauge polyethylene tubing. All glycine solutions were prepared in MBS or MBS +2.0 or 2.5 mM tricine. When maximally-effective concentrations of glycine were applied, applications lasted for 15 s and were followed by 10 min washouts with MBS to allow for complete receptor resensitization. For experiments using submaximal concentrations of glycine, concentrations that yielded 5% of the maximally-effective glycine response (EC_5) were applied for 45 s followed by 3 min washouts with MBS to allow for complete receptor resensitization. Data were acquired at a rate of 1 kHz using a Powerlab 4/30 digitizer using LabChart version 7 software (ADInstruments, Bella Vista, NSW, Australia).

2.4. 4–Cadmium and zinc concentration determination

Zinc and cadmium concentrations were determined in MBS and distilled water using a quadrupole-based Agilent 7500ce inductively-coupled plasma mass spectrometer (ICP-MS) at the Jackson School of Geosciences Isotope Geochemistry Facility at the University of Texas at Austin. Solutions were diluted as necessary in 2% HNO_3 before analysis.

2.5. Data analysis

Peak currents were measured and used in data analysis. Currents generated under the various experimental conditions were normalized against currents generated by the indicated control applications and expressed as mean ± S.E.M. of the percent of control generated current (Sections 3.1 and 3.2) or percent change from control generated current (Section 3.3). Statistically significant differences among experimental conditions were determined using one- or three-way ANOVAs and post hoc tests, as indicated. SigmaPlot version 11.0 (Systat Software, San Jose, CA) was used for statistical testing.

3. Results

3.1. Type of vial containing glycine does not affect degree of contaminating zinc-mediated GlyR enhancement

To determine if various vials commonly used for the preparation of agonist solutions contain different amounts of contaminating zinc sufficient to affect GlyR currents, glycine solutions were prepared in three different vials in the presence of the zinc-chelating agent tricine or without: Fisher glass screw-thread vials (Cat. No. 03-339-22J), National Scientific silanized glass vials (Cat. No. B7999-S3), or BD Falcon™ polypropylene tubes (Cat. No. 352096). Low concentrations of zinc (below 10 μM) left-shift glycine concentration-response curves of α1-containing GlyR, with the greatest enhancing effects of zinc seen at low concentrations of glycine (Laube et al., 1995). We therefore tested, in different types of vials, concentrations of glycine that elicited 5% of maximally-effective glycine responses (EC_5).

EC_5 concentrations of glycine in buffer containing 2.5 mM tricine were determined in Fisher glass vials and used as controls against which all other experimental conditions were normalized (Fig. 1A and B). Fig. 1A shows a sample tracing of successive 45 s applications of EC_5 glycine from solutions made up in glass, silanized glass or polypropylene vials, with or without 2.5 mM tricine. Control applications of EC_5 glycine + 2.5 mM tricine in glass were interspersed throughout to account for any drift in EC_5 glycine-mediated responses. For all vials, GlyR currents mediated by EC_5 glycine in MBS without tricine were consistently higher than currents generated by EC_5 glycine in MBS + 2.5 mM tricine [$F(4,19) = 21.77$, $p < 0.001$] (Fig. 1B). However, Student–Newman–Keuls post hoc tests revealed no significant differences in currents generated by EC_5 glycine in MBS among the different vials used, in either the absence or presence of tricine (Fig. 1B). As previously shown by McCracken et al. (2010), chelation of zinc by tricine does not affect currents mediated by saturating concentrations of glycine (Fig. 1C). Further, vial choice does not significantly affect currents mediated by saturating (10 mM) concentrations of glycine [$F(4,8) = 1.77$, $p > 0.19$] (Fig. 1C).

3.2. Washing agonist-solution vials does not affect the degree of contaminating zinc-mediated GlyR enhancement

We next wished to determine whether zinc was present on the surfaces of vials and whether washing them prior to the preparation of EC_5 glycine solutions would decrease contaminating zinc-mediated GlyR enhancement. Glass and polypropylene vials were either: (1) not washed, (2) washed 5 times with deionized H_2O (diH_2O), (3) washed 50 times with diH_2O, or (4) soaked in MBS + 2.5 mM tricine for 10 min before immediate drying and preparation of EC_5 glycine. GlyR responses elicited by EC_5 glycine prepared in these were compared with EC_5 glycine solutions prepared in corresponding unwashed vials with MBS +2.5 mM tricine. The various washing procedures did not affect the degree of contaminating zinc-mediated GlyR enhancement for polypropylene [$F(3,19) = 0.03$, $p > 0.99$] or glass [$F(3,11) = 0.02$, $p > 0.99$] vials (Fig. 2A and B).

3.3. Polystyrene but not glass serological pipets contain contaminating zinc that significantly affects EC_5 glycine-mediated GlyR currents

During the course of conducting these studies we observed that the first EC_5 glycine solution made using a polystyrene serological pipet to transfer the MBS buffer to vials seemed to produce larger GlyR currents than subsequent EC_5 glycine solutions made using the same pipet to transfer MBS. Further, it appeared that this