0026-895X/94/061156-04$03.00/0

MOLECULAR PHARMACOLOGY, 46:1156–1159, 1994.

Biphasic Modulation of the Strychnine-Sensitive Glycine Receptor by Zn^{2+}

AARON B. BLOOMENTHAL, ELAINE GOLDWATER, DOLAN B. PRITCHETT, and NEIL L. HARRISON

Department of Anesthesia and Critical Care (A.B.B., E.G., N.L.H.), Department of Pharmacological and Physiological Sciences (N.L.H.), The University of Chicago, Chicago, Illinois 60637, and Department of Pharmacology (D.B.P.), University of Pennsylvania, Philadelphia, Pennsylvania 19105.

Received June 30, 1994; Accepted September 13, 1994

SUMMARY

The effects of extracellular applications of Zn^{2+} ions on the strychnine-sensitive glycine receptor were studied in cultured rat spinal cord neurons and with recombinant glycine receptors expressed in human embryonic kidney 293 cells. Nanomolar concentrations of Zn^{2+} enhanced the chloride ion current in response to brief applications of 100 μM glycine. The enhancement of glycine responses increased from 20 nM to 1 μM Zn^{2+}. Higher concentrations of Zn^{2+} caused a reversal of the potentiation, followed by progressive inhibition of the glycine response up to ~20–50 μM Zn^{2+}. The biphasic modulation by Zn^{2+} appeared essentially identical in native and recombinant glycine receptors. Biphasic Zn^{2+} modulation was observed both with picrotoxin-insensitive heteromeric ($\alpha 2/\beta$) receptors and with picrotoxin-sensitive homomeric receptors consisting only of $\alpha 2$ subunits. This suggests that the α subunit alone is sufficient for formation of two distinct Zn^{2+} binding sites on the glycine receptor. The demonstration of Zn^{2+} modulation of the strychnine-sensitive glycine receptor is of potential physiological importance, in view of the likely range of subsynaptic Zn^{2+} concentrations to which the receptor is exposed.

Zn^{2+} ions are found throughout the mammalian central nervous system, being concentrated in nerve terminals, especially in the mossy fiber terminals of the hippocampus (1). Zn^{2+} is an essential element for the normal development of the nervous system (2) but, paradoxically, is suspected to act as a neurotoxin at higher concentrations (3, 4). There is a great deal of evidence in favor of Zn^{2+} as an endogenous neuromodulator. Zn^{2+} is released from the mossy fiber terminals of the hippocampus and causes significant alterations in the excitability of single neurons and local networks (5–8). An explanation for these neuromodulatory actions of Zn^{2+} may be found in its interactions with an extensive array of voltage- and ligand-gated ion channels (reviewed in Ref. 9), including the $GABA_A$ receptor (10–12) and the NMDA receptor (10, 13). Modulation of the $GABA_A$ receptor by Zn^{2+} is critically dependent upon the subunit composition of the receptor (14). A close relative of the $GABA_A$ receptor within the 'subfamily' of ligand-gated chloride channels is the glycine receptor (15), which is a heteropentameric receptor composed of α and β subunits (16). The glycine receptor is found primarily in the spinal cord and brainstem, where glycine serves as an important inhibitory neurotransmitter. We initially studied the effects of Zn^{2+} on native glycine receptors in cultured spinal neurons. We also investigated the modulation by Zn^{2+} of recombinant homomeric and heteromeric glycine receptors in a heterologous expression system.

Materials and Methods

Cell culture. Spinal neurons from the embryonic rat (embryonic days 16–19) were dissociated and maintained in culture using previously published methods (17, 18). HEK 293 cells were obtained from the American Type Culture Collection and maintained in culture as described previously (19).

Plasmid preparation, purification, and transfection. The human glycine receptor $\alpha 2$ subunit (20) and the rat glycine receptor β subunit (21) were expressed using the mammalian expression vector pCIS2. The vector contains one copy of the strong promoter from cytomegalovirus and a polyadenylation sequence from simian virus 40. HEK 293 cells were transfected using the calcium phosphate precipitation technique (22). Two to 3 μg of $\alpha 2$ DNA were used for homomeric receptors; for co-transfections, 1–2 μg of $\alpha 2$ and 10–20 μg of β subunit DNA were used; this 1:10 ratio has been previously demonstrated to be optimal.

Electrophysiology. Recordings were made using the whole-cell patch-clamp technique (23). Patch pipettes contained 145 mM *N*-methyl-D-glucamine hydrochloride, 5 mM K_2ATP, 1.1 mM EGTA, 2 mM $MgCl_2$, 5 mM HEPES/KOH, pH 7.2, and 0.1 mM $CaCl_2$. Pipette resistance was 4–5 MΩ. The extracellular medium contained 145 mM NaCl, 3 mM KCl, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, 6 mM D-glucose, and 10 mM HEPES/NaOH, pH 7.4. Spinal neurons or isolated HEK 293 cells

This research was supported in part by National Institutes of Health NIEMS Grant GM00623 (to N.L.H.).

ABBREVIATIONS: GABA, γ-aminobutyric acid; HEK, human embryonic kidney; NMDA, *N*-methyl-D-aspartate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

were voltage clamped at −60 mV. Glycine (100 μM) was applied to the region of the cell body by pressure (6–12 psi) from blunt-tipped micropipettes. Zn^{2+} was applied via the extracellular medium. Responses were low-pass filtered at 2 kHz and digitized (TL-1-125 interface; Axon Instruments, Foster City, CA). Numerical data are presented throughout as mean ± standard error of Δ, where Δ is the percentage change in the amplitude of the glycine response observed with drug, relative to control. ATP was from Calbiochem, strychnine, bicuculline, and picrotoxin from Research Biochemicals (Natick, MA), and all other chemicals from Sigma Chemical Co. (St. Louis, MO).

Results

The effects of Zn^{2+} were studied in cultured rat spinal neurons. Glycine-activated currents were very sensitive to inhibition by strychnine, being reduced in amplitude [$\Delta = -94 \pm 4\%$ ($n = 4$) with 100 nM strychnine] (Fig. 1a). The glycine responses were insensitive to the GABA antagonist bicuculline [$\Delta = +2 \pm 9\%$ ($n = 4$) with 20 μM bicuculline] (Fig. 1b). Currents through native glycine receptors were insensitive to picrotoxin [$\Delta = +7 \pm 6\%$ ($n = 4$) with 1 μM picrotoxin; $\Delta = +6 \pm 7\%$ ($n = 4$) with 10 μM picrotoxin] (Fig. 1, c and d). Glycine-activated currents were strongly potentiated by low concentrations of Zn^{2+} [$\Delta = +183 \pm 22\%$ ($n = 5$) with 1 μM Zn^{2+}] (Fig. 2a). As the Zn^{2+} concentration was increased into the micromolar range, the effect of Zn^{2+} became highly variable between 2 and 10 μM before eventually reversing; in fact, at higher micromolar concentrations of Zn^{2+} the glycine response was consistently strongly inhibited [$\Delta = -78 \pm 2\%$ ($n = 4$) with 50 μM Zn^{2+}] (Fig. 2b).

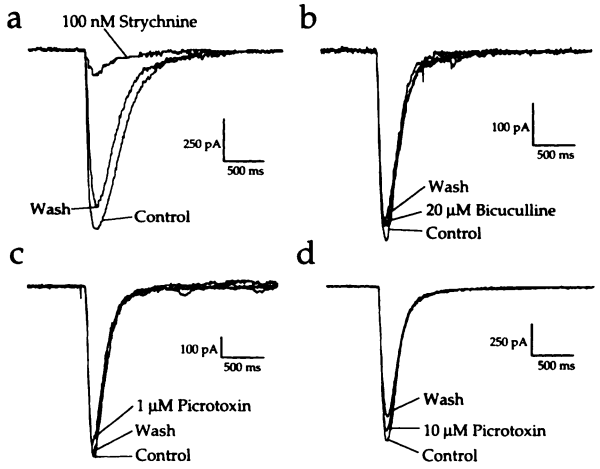

Fig. 1. Pharmacology of native glycine receptors in rat spinal neurons. a, Currents through native glycine receptors are blocked by 100 nM strychnine. b, Native glycine receptors are insensitive to block by 20 μM bicuculline. c, Glycine responses are unaltered by 1 μM picrotoxin. d, Glycine responses are also unaltered by 10 μM picrotoxin. Inward currents were activated by brief pressure application of 100 μM glycine to the cell body of a cultured rat spinal neuron, before, during, and after application of antagonist. In all figures, glycine-evoked inward currents were recorded under voltage clamp at −60 mV.

Application of glycine to HEK 293 cells transfected with the α2 subunit cDNA resulted in large inward currents in 20–30% of the cells tested. Maximal currents in these cells were frequently on the order of 2–5 nA. As expected, and like the glycine-activated currents in spinal neurons, these currents reversed in polarity near 0 mV under these experimental conditions ($E_{gly} = -7.3 \pm 2.5$ mV in a subset of four cells). Glycine-activated currents through these homomeric channels were also very sensitive to inhibition by strychnine [$\Delta = -99.5 \pm 0.3\%$ ($n = 6$) with 100 nM strychnine] (Fig. 3a). The α2 receptor responses were insensitive to the GABA antagonist bicuculline [$\Delta = -2 \pm 3\%$ ($n = 4$) with 20 μM bicuculline] (Fig. 3b). As reported previously, (21), currents through homomeric glycine receptors were also rather sensitive to picrotoxin [$\Delta = -45 \pm 3\%$ ($n = 4$) with 1 μM picrotoxin] (Fig. 3c) and were almost completely blocked by 10 μM picrotoxin [$\Delta = -92 \pm 1\%$ ($n = 5$) with 10 μM picrotoxin] (Fig. 3d). Glycine-activated currents in these recombinant homomeric receptors were also potentiated by nanomolar concentrations of Zn^{2+}, being increased in amplitude with concentrations as low as 100 nM Zn^{2+} [$\Delta = +26 \pm 5\%$ ($n = 4$) with 100 nM Zn^{2+}] and doubled in amplitude with 500 nM Zn^{2+} [$\Delta = +103 \pm 26\%$ ($n = 4$) with 500 nM Zn^{2+}] (Fig. 3e). As the Zn^{2+} concentration was increased into the micromolar range, the effect of Zn^{2+} became less consistent before reversing around 5 μM; in fact, the glycine response was consistently almost completely blocked at 50 μM Zn^{2+} [$\Delta = -86 \pm 11\%$ ($n = 5$) with 50 μM Zn^{2+}] (Fig. 3f). Neither the enhancement by 1 μM Zn^{2+} nor the inhibition by 50 μM Zn^{2+} was associated with any changes in the reversal potential for the glycine-induced currents.

Finally, the effects of Zn^{2+} were also studied with heteromeric glycine receptors, using co-transfection of 293 cells with both α2 and β subunits. Glycine-activated currents were again blocked by 100 nM strychnine but were insensitive to bicuculline. As reported previously (24), addition of the β subunit rendered the expressed receptors relatively insensitive to picrotoxin [$\Delta = -2 \pm 6\%$ ($n = 4$) with 1 μM picrotoxin; $\Delta = -63 \pm 16\%$ ($n = 4$) with 10 μM picrotoxin] (Fig. 4, a and b). Nanomolar Zn^{2+} also potentiated currents through heteromeric glycine receptors (Fig. 4c), but at >20 μM Zn^{2+} the glycine response was completely inhibited [$\Delta = -96 \pm 2\%$ ($n = 6$) with 50 μM Zn^{2+}] (Fig. 4d).

A full concentration-response curve was constructed for modulation of homomeric α2 glycine receptors by Zn^{2+}. These data show clearly the biphasic nature of the interaction (Fig. 5). Potentiation of the glycine response first becomes significant at 20 nM and increases to reach a maximum near 1 μM Zn^{2+}.

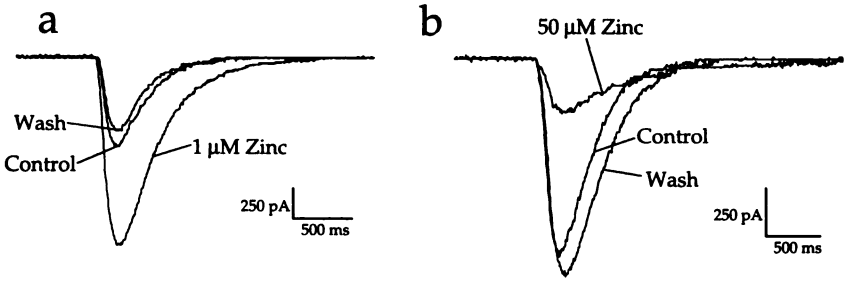

Fig. 2. Enhancement by micromolar Zn^{2+} of currents evoked by glycine at native glycine receptors and block of glycine responses by higher Zn^{2+} concentrations. a, Zn^{2+} enhancement of pharmacological responses to glycine in spinal neurons. b, Zn^{2+} antagonism of pharmacological responses to glycine in spinal neurons.

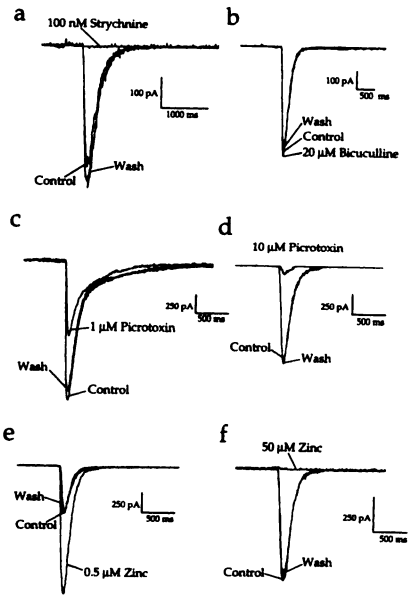

Fig. 3. Pharmacology of homomeric $\alpha 2$ glycine receptors. a, Currents through glycine-activated channels are blocked by 100 nM strychnine. b, Homomeric glycine receptors are insensitive to block by 20 μM bicuculline. c, Glycine responses of homomeric receptors are inhibited by 1 μM picrotoxin. d, Glycine responses of homomeric receptors are completely blocked by 10 μM picrotoxin. e, Zn^{2+} at 500 nM enhances glycine responses of homomeric receptors. f, Zn^{2+} at 50 μM blocks glycine responses of homomeric receptors. Here and in Fig. 4, inward currents were activated by brief pressure application of 100 μM glycine to the cell body of an individual HEK 293 cell, before, during, and after application of antagonist.

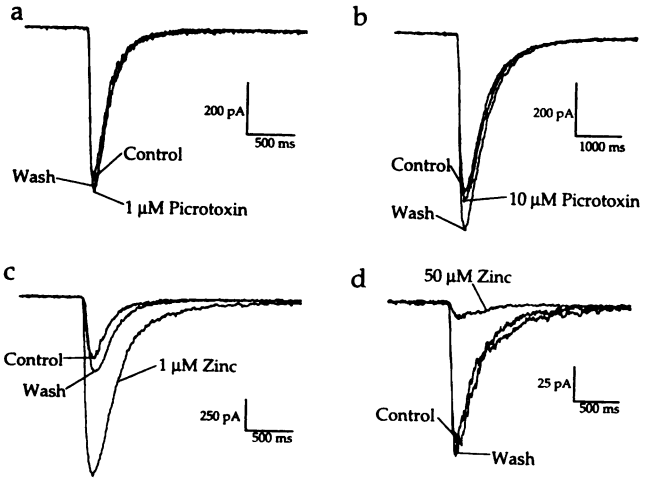

Fig. 4. Pharmacology of heteromeric $\alpha 2\beta$ glycine receptors. a, Currents through recombinant $\alpha 2\beta$ glycine receptors are insensitive to 1 μM picrotoxin. b, Recombinant heteromeric glycine receptors are virtually insensitive to block by 10 μM picrotoxin. c, Zn^{2+} at 500 nM enhances responses to glycine at $\alpha 2\beta$ receptors. d, Zn^{2+} at 50 μM strongly inhibits responses to glycine at recombinant $\alpha 2\beta$ receptors.

Above 2 μM, the inhibitory effects of Zn^{2+} begin to predominate, with complete block being observed at $\geq$50 μM Zn^{2+}.

Discussion

The results of this study show clearly that Zn^{2+} has biphasic actions both on the native glycine receptor in rat spinal cord neurons and on recombinant glycine receptors expressed in HEK 293 cells. Homomeric receptors formed from the human glycine receptor $\alpha 2$ subunit alone retained sensitivity to Zn^{2+}. We suggest that the opposing actions of Zn^{2+} are separate processes, resulting from the interactions of the metal ion with two distinct binding sites, with different affinities, on the channel protein.

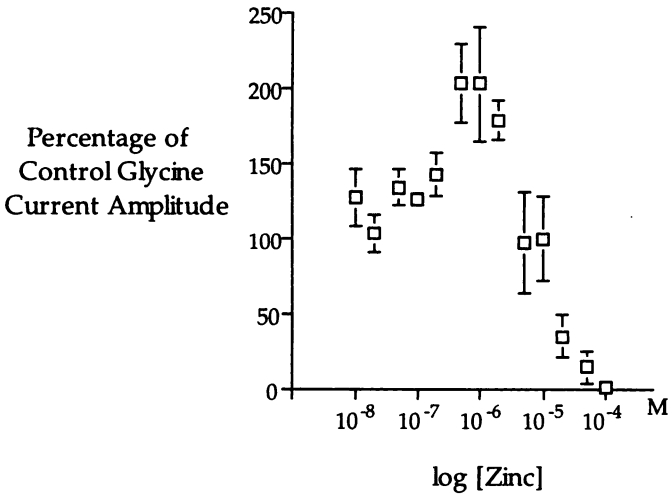

Fig. 5. Biphasic and concentration-dependent effects of Zn^{2+} on human glycine receptors. The concentration-response relationship for the effects of Zn^{2+} on recombinant glycine receptors consisting only of the human $\alpha 2$ subunit is shown. The percentage change in response amplitude, relative to control responses, is plotted against the logarithm of extracellular Zn^{2+} concentration. Each *point* represents the mean $\pm$ standard error of four to 12 experiments.

The block of glycine receptors by Zn^{2+} was the less surprising finding, in view of the considerable degree of homology between the α subunits of the $GABA_A$ receptor and those of the glycine receptor. $GABA_A$ receptors composed of α and β subunits are inhibited by Zn^{2+} with an IC_{50} of ~1 μM. Coexpression of the γ subunit with α and β subunits renders these receptors much less sensitive to Zn^{2+} (14). The IC_{50} for the Zn^{2+} reduction of glycine responses is difficult to estimate, because of the potentiating effect of lower Zn^{2+} concentrations, but presumably must be on the order of ~5 μM. This evidence suggests that the site responsible for antagonism of GABA actions at $\alpha\beta$ heteromeric $GABA_A$ receptors might also reside on the α subunit. This hypothesis has been difficult to test directly for the $GABA_A$ receptor, because of the inefficient assembly of homomeric $GABA_A$ receptors in mammalian cells (25).

The potentiating action of nanomolar Zn^{2+} was the more unexpected finding. Few modulatory agents potentiate glycine currents, in contrast to the constellation of substances that promote currents through $GABA_A$ receptor chloride channels. Presumably, the potentiating action of Zn^{2+} in each case is mediated by a quite distinct binding site, compared with the inhibitory effect. No such potentiating effects have been observed with $GABA_A$ receptors, although a biphasic action of Zn^{2+} was recently reported for ATP-gated channels, i.e., P_2 purinergic receptors (26), and for a splice variant of the NR1 sububnit of the NMDA receptor (13, 27). In contrast, lowering the Zn^{2+} concentration into the nanomolar range does not produce potentiating effects on $\alpha\beta$ or $\alpha\beta\gamma$ $GABA_A$ receptors expressed in HEK 293 cells.[1] This indicates that the positive

[1] T. A. Verdoorn, personal communication.

modulation by Zn^{2+} is exerted at a site not shared with the $GABA_A$ receptor subunits but perhaps shared with other ligand-gated channels.

Native glycine receptors are believed to be heteromeric pentamers consisting of three α subunits together with two β subunits (16). The glycine receptor β subunit has a distinctly different sequence within the M2 segment, compared with the three α subunit isoforms (20). These alterations in M2 result in a channel with reduced sensitivity to block by picrotoxin (24) and a reduced single-channel conductance of ~45 pS, relative to the high conductance (~110 pS) of the α homomeric channels (28). In the present study, we used the decrease in picrotoxin sensitivity to confirm successful expression of the β subunit by co-transfection in our experiments with α and β subunit cDNAs. Expression of heteromeric receptors containing the β subunit appeared to have little influence on the effects of zinc. We must conclude either that both α and β subunits have identical Zn^{2+} binding sites or that the binding sites reside on the α subunit and are unaffected by the presence of the β subunit. This contrasts with the inhibitory Zn^{2+} binding site on the $GABA_A$ receptor, which is located on either α or β subunits (or both) but is altered in receptors containing the γ subunit. Site-directed mutagenesis of the glycine receptor subunits should yield useful information concerning residues involved in Zn^{2+} actions on both glycine and $GABA_A$ receptor subunits. In this context, the parallel between the strychnine-sensitive glycine receptor and the NMDA receptor is particularly interesting, because the NMDA receptor is also known to possess a requirement for glycine as coagonist.

In the hippocampus Zn^{2+} concentrations in the high micromolar range have been estimated (1), but in most other regions of the nervous system, in which Zn^{2+} is believed to be less concentrated, subsynaptic Zn^{2+} concentrations may be a great deal lower. Circulating plasma and cerebrospinal fluid free Zn^{2+} concentrations are on the order of 100–200 nM (29), although Zn^{2+} concentrations in the spinal cord are unknown. It is possible that the modulatory effects of nanomolar to micromolar concentrations of Zn^{2+} on glycine receptors are of physiological relevance and that Zn^{2+} acts at glycine receptors in the spinal cord and brainstem to regulate the efficiency of inhibitory transmission.

Acknowledgments

The authors are indebted to Prof. Heinrich Betz for the gift of rat glycine receptor β subunit cDNA, to Dr. Peter Schofield for the human glycine receptor $\alpha 2$ subunit cDNA, and to Andrew Spellman and Olgica Bakajin for excellent technical support.

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Send reprint requests to: Neil L. Harrison, Department of Anesthesia and Critical Care, Box 428, The University of Chicago, 5841 S. Maryland Ave., Chicago, IL 60637.