Published in final edited form as:

Neuropharmacology. 2010 March ; 58(3): 676–681. doi:10.1016/j.neuropharm.2009.11.001.

Zinc Enhances Ethanol Modulation of the $\alpha 1$ Glycine Receptor

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Summary

Glycine receptor function mediates most inhibitory neurotransmission in the brainstem and spinal cord and is enhanced by alcohols, volatile anesthetics, inhaled drugs of abuse, and endogenous compounds including zinc. Because zinc exists ubiquitously throughout the brain, investigations of its effects on the enhancement of GlyR function by alcohols and anesthetics are important to understanding the effects of these agents *in vivo*. In the present study, the effects of zinc plus ethanol, pentanol, or isoflurane were tested on homomeric $\alpha 1$ glycine receptors to determine if concurrent applications of physiological concentrations of zinc with each of these modulators changed the magnitude of their effects. Homomeric $\alpha 1$ glycine receptors were expressed in *Xenopus laevis* oocytes, and the two-electrode voltage clamp technique was used to measure glycine-mediated currents in the presence of combinations of zinc with ethanol, pentanol or isoflurane. The combined effects of zinc plus ethanol were greater than the sum of the effects produced by either compound alone. However, this was not seen when zinc was combined with either pentanol or isoflurane. Chelation of zinc by tricine decreased the effects of submaximal, but not maximal, concentrations of glycine, and diminished the magnitude of ethanol enhancement observed. These findings suggest a zinc/ethanol interaction at the $\alpha 1$ GlyR that results in the enhancement of the effects of ethanol action on GlyR function.

Keywords

Ethanol; zinc; glycine receptor; electrophysiology; *Xenopus* oocytes

1. Introduction

Glycine receptors (GlyRs) are the primary mediators of inhibitory neurotransmission in the brain stem and spinal cord (Legendre, 2001) and anion-conducting members of the nicotinic acetylcholine superfamily of ligand-gated ion channels. Two classes of GlyR subunits have been identified (α and β); four α subunits, with 80-90% sequence identity among subunits, and a single β subunit, possessing ~50% sequence identity with the α subunits (Lynch, 2004). The α subunits can express homomERICALLY, or heteromERICALLY with β subunits, to produce

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functional channels. In addition to the spinal cord and medulla, GlyRs are found in several brain regions including the hippocampus (Fatima-Shad and Barry, 1993), nucleus accumbens (Molander and Söderpalm, 2005), cerebellum (Takahashi et al., 1992) and also in the olfactory bulb (van den Pol and Gorcs, 1988).

A number of positive modulators of GlyR function exist, including alcohols, volatile anesthetics and inhaled drugs of abuse (Mihic et al. 1997, Beckstead et al. 2000). Zinc, which is found endogenously in both free and protein-bound forms, also affects GlyR function, but in a biphasic manner. Concentrations lower than 10 μ M have enhancing effects, whereas concentrations greater than 10 μ M inhibit GlyR function (Harvey et al. 1999, Laube et al. 2000). Zinc concentrations in the brain exceed those present in other organs, although most zinc is protein-bound (Mathie et al. 2006). In its free or rapidly exchangeable form, zinc exists in cerebrospinal fluid at basal concentrations ranging from approximately 5-25 nM (Frederickson et al. 2006), and is predicted to remain at concentrations below 10 μ M following presynaptic release from GABAergic, glutamatergic, or glycinergic terminals (Frederickson et al. 2001).

Because zinc exists ubiquitously throughout the brain, investigations of its effects on the enhancement of GlyR function by alcohols and anesthetics are important to understanding the effects of these agents *in vivo*. In the present study, we examined whether co-applying low, physiologically-relevant, concentrations of zinc with either ethanol (EtOH), pentanol, or isoflurane would result in augmented effects of these modulators on α 1 GlyR function.

2. Methods

2.1 Reagents

All reagents were purchased from Sigma-Aldrich (St. Louis, MO). *Xenopus laevis* were obtained from Xenopus Express (Brooksville, FL).

2.2 Oocyte Isolation and cDNA Injection

Partial ovariectomies were performed on sexually mature female *Xenopus laevis* and ovary fragments were placed in isolation media (108 mM NaCl, 2 mM KCl, 1 mM EDTA, 10 mM HEPES). Stage IV and V oocytes were manually extracted from the thecal and epithelial membranes with forceps under a light microscope. In order to remove the follicular membrane, isolated oocytes were immersed in 0.5 mg/ml collagenase in collagenase buffer (83 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$) for 10 minutes and then were transferred into Modified Barth's Solution (MBS) (88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 10 mM HEPES, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.91 mM $CaCl_2$) for cDNA injection.

The animal pole of each oocyte was injected with (1.5 ng / 30 nl) wild type human α 1 GlyR cDNA using an automated injector (Drummond Nanoinject II, Broomall, NY). Injected oocytes were incubated singly in 96-well plates filled with incubation media (MBS, 2 mM Na pyruvate, 0.5 mM theophylline, 10 U/ml penicillin, 10 mg/l streptomycin, 50 mg/l gentamycin) and were stored in the dark at 18°C. Glycine receptor expression was observed as soon as one day after cDNA injection.

2.3 Two-Electrode Voltage Clamp Electrophysiology

Oocytes were impaled in the animal poles with two high-resistance (>1 M Ω) glass electrodes containing 3N KCl and were voltage-clamped at -70mV using a Warner OC-725C oocyte clamp (Warner Instruments, Hamden, CT). A Masterflex USA peristaltic pump (Cole-Parmer Instrument Corporation, Vernon Hills, IL) was used to deliver MBS to oocytes via bath perfusion at a rate of 2 ml/min. Clamping currents were recorded on a chart recorder (Cole-

Parmer Instrument Corporation, Vernon Hills, IL) interfaced to the oocyte voltage-clamp apparatus.

2.4 Tricine Chelation of Zinc

Glycine concentration-response curves were generated for oocytes expressing wildtype $\alpha 1$ GlyRs in presence and in the absence of the zinc-chelating agent tricine. The effects of a series of glycine concentrations (10 μ M - 10 mM) were tested, and the glycine concentration that produced the greatest current was determined to be the maximally-effective concentration. The currents elicited by all other concentrations of glycine tested were recorded as percentages of that maximal glycinergic effect.

In addition, the effects of zinc chelation on ethanol modulation of $\alpha 1$ GlyRs were investigated. First 10 mM glycine was applied to oocytes expressing $\alpha 1$ GlyRs to determine a maximal effect of glycine; this current was then subsequently used to identify the EC₅₋₁₀ concentration of glycine, producing 5-10% of a maximal glycinergic effect. The enhancing effects of a series of EtOH concentrations (20 mM, 50 mM, and 200 mM) on EC₅₋₁₀ glycine currents were also measured. Tricine at a concentration of 10 mM was then bath-applied for 2 min and the maximal glycine effect determined again. The concentration of glycine shown to be EC₅₋₁₀ in the absence of tricine was then re-applied. Any decrease in current would be hypothesized to be due to tricine chelation of trace free zinc contamination, which is typically found in the 100 nM – 1 μ M range in many buffers (Kay, 2004). Lastly, 20 mM, 50 mM, and 200 mM ethanol modulation of GlyR function using that EC₅₋₁₀ glycine concentration was also assessed in the continued presence of tricine.

2.5 Sustained Perfusion of Zinc with Ethanol, Pentanol or Isoflurane

Oocytes were perfused with 10 μ M glycine (~EC₂) for 2 minutes, followed by a 30 second application of 200 mM EtOH that was concurrently applied with 10 μ M glycine. After a washout period, during which 10 μ M glycine perfusion persisted, 50 nM zinc was perfused with glycine for 2 min and then 200 mM EtOH was added concurrently with zinc and glycine for 30 seconds. This experimental protocol was also used to test the interactions of zinc with the longer chain alcohol pentanol, as well as the volatile anesthetic isoflurane. Either 0.6 mM isoflurane or 2.9 mM pentanol were first applied in the absence and then in the presence of 50 nM zinc as described above. Pentanol was tested at a concentration corresponding to 1 MAC (an anesthetic ED₅₀), while isoflurane was tested at a concentration equivalent to 2 MAC.

2.6 Inductively Coupled Plasma Spectrometry

Using inductively-coupled plasma spectrometry, MBS sample solutions were tested for zinc contamination against a standard curve. The reliable detection limit was approximately 30 nM, and all non-zinc-treated solutions tested had free zinc levels at or below this limit.

2.7 Data Analysis

Responses were assessed as changes in peak heights of chloride currents measured from chart recorder tracings. Data were tested for statistical differences using paired t-tests or two-way ANOVA, as indicated. Statistical significance was determined at $p < 0.05$.

3. Results

To examine the effects of physiological-relevant concentrations of zinc on the enhancement of GlyR function by alcohols and anesthetics, we used two approaches. First, the zinc chelator tricine was included in solutions to remove any trace levels of zinc, and then in a second approach, physiologically-relevant concentrations of zinc were added to our perfusion

solutions. The effects of zinc on GlyR modulation by ethanol, pentanol and isoflurane were tested.

Tricine at a concentration of 10 mM was used to chelate free zinc in MBS buffer. Glycine concentration-response curves were generated in the absence and presence of tricine. Tricine-containing buffer did not affect the currents elicited by a maximally-effective concentration of glycine [$t(10) = 0.29$, $p > 0.78$] but did significantly decrease the currents seen in the presence of EC5-10 glycine [$t(3) = 4.6$, $p < 0.02$] (Fig. 1). Tricine also significantly reduced the magnitude of 20-200 mM EtOH enhancement of EC5-10 glycine responses [$F(1,25) = 17.3$, $p < 0.001$] (Figs. 2A & 2B). In a zinc control experiment, application of 1 μ M zinc in tricine-free buffer enhanced the effect of EC5-10 glycine by approximately 200%, but this concentration of zinc had no effect when it was applied in the presence of 10 mM tricine [$t(5) = 6.6$, $p < 0.001$] (Fig. 2C), indicating the chelation of zinc by tricine.

In another test of zinc enhancement of the actions of ethanol at GlyRs, 200 mM EtOH was first tested for its enhancing effects in the presence of 10 μ M glycine and then re-tested during a concurrent application of 50 nM zinc with 10 μ M glycine. A concentration of 10 μ M glycine produced minimal desensitization during sustained application (Fig. 3A). When ethanol was co-applied it enhanced the effect of glycine; the same was true for zinc. Once a plateau was reached in the zinc effect, ethanol was co-applied with zinc to determine the effects of these two compounds applied together (Fig. 3A). The amount of absolute current produced by the application of 200 mM EtOH was significantly greater when it was co-applied with 50 nM zinc [$t(10)=2.5$, $p<0.03$] (Fig. 3B).

In order to determine if zinc could also interact with other modulators to increase their effects on GlyR function, the effects of either 0.6 mM isoflurane or 2.9 mM pentanol with 50 nM zinc were next tested in the same manner as ethanol had been in Fig. 3. Each of these compounds was first individually applied with 10 μ M glycine and then re-applied during a concurrent 50 nM zinc application. The combined effects of zinc plus isoflurane were not synergistic; in fact the amplitude of the current produced by isoflurane did not change whether it was applied with or without zinc (Fig. 4A). Co-application of 50 nM zinc with 0.6 mM isoflurane did not increase the amount of absolute current that was produced by isoflurane alone [$t(3)= 0.09$, $p>0.93$] (Fig. 4B). Similarly, the amount of current produced by 2.9 mM pentanol was not significantly different when applied in the presence or absence of a 50 nM zinc [$t(2)=0.06$; $p> 0.95$] (Fig. 4B).

4. Discussion

Most investigations of modulators of channel function involve the sole application of the modulatory compound of interest, which may inadequately represent *in vivo* conditions. For example, free zinc exists in the CNS at concentrations known to affect GlyR function, which led us to speculate whether it might affect the enhancing effects of other GlyR allosteric modulators. In experiments in which zinc and ethanol were concurrently applied, low physiologically-relevant concentrations of zinc enhanced the magnitude of the effects of ethanol on wild-type $\alpha 1$ GlyR function. However, zinc did not affect the enhancement of GlyR function by either isoflurane or pentanol. In order for zinc and another modulatory compound to interact at the level of a single receptor, both compounds must be bound simultaneously to that receptor. Consequently, the lack of a difference seen when isoflurane or pentanol were co-applied with zinc versus when they were individually applied may reflect low probabilities of any given GlyR simultaneously binding both isoflurane, or pentanol, and zinc. The effects of these compounds on GlyR function are seen at relatively lower concentrations than the concentration of ethanol required to produce appreciable effects on GlyR function. In

agreement with these findings is a recent study by Jenkins et al. (2008) in which the effects of 75 μ M isoflurane and 100 nM zinc on GlyR function were determined to be additive.

Several lines of evidence highlight the physiological relevance of free zinc in the central nervous system (CNS) and its ability to modulate GlyR function there. For example, the significance of zinc to GlyR function was recently demonstrated in knockin mice carrying a D80A mutation in the GlyR α 1 subunit gene (*Glr1*) (Hirzel et al., 2006). Aspartate-80 is an important residue for the high-affinity binding of zinc, leading to enhancement of GlyR function (Laube et al., 2000). Mice homozygous for *Glr1* (D80A) exhibit phenotypes analogous to human startle disease, and *in vitro* studies of spinal neurons and brainstem slices from these animals revealed significant impairments in the enhancement of spontaneous glycinergic currents by zinc (Hirzel et al., 2006). In addition, basal concentrations of zinc found in the CNS have also been shown to be sufficient for the prolongation of the decay phase of glycinergic miniature inhibitory postsynaptic currents (Suwa et al., 2001). Our tricaine data support the hypothesis that low baseline concentrations of zinc reduce the EC50 of glycine concentration-response curves without enhancing maximally-effective glycinergic currents. However, also in agreement with Hirzel et al. (2006), the binding of zinc is not obligatory for GlyR function, since glycine-mediated currents were still seen in the presence of 10 mM tricaine. Tricaine is a widely-accepted and preferred chelator of zinc as reviewed in Paoletti et al. (2009). Although zinc is present throughout the brain, it is often located in the vesicles of GABAergic or glutamatergic neurons. Specifically, zinc-enriched GABAergic terminals are found in the cerebellum (Wang et al. 2002), and glutamatergic terminals containing vesicular zinc predominate in the cerebral cortex, amygdalar nuclei, olfactory bulb, and the hippocampal formation (Frederickson & Bush, 2001), most of which notably also contain GlyRs.

Although the exact sites and mechanisms for zinc modulation of GlyR function are not completely understood, several amino acids in the *N*-terminal domain of the α 1 subunit responsible for the enhancing and inhibiting effects of zinc on GlyR function have been identified. The potentiating effects of zinc, generally seen at concentrations less than 10 μ M, require high-affinity binding to amino acids including aspartate-80, threonine-151, glutamate-192, aspartate-194 and histidine-215 (Laube et al. 2000; Miller et al. 2005b). Additional residues of the GlyR α 1 subunit, in particular histidine-107, histidine-109, threonine-112 and threonine-133 are thought to contribute to lower-affinity binding sites and are necessary for inhibition of GlyR function by zinc at concentrations greater than 10 μ M (Harvey et al., 1999; Laube et al., 2000; Miller et al., 2005a). The importance of zinc binding at these lower affinity (GlyR inhibitory) sites is illustrated in instances of ischemia, seizure, trauma, and neurodegeneration, during which zinc levels are estimated to peak in the brain at concentrations greater than 100 μ M (Choi & Koh, 1998; Doraiswamy & Finefrock, 2004).

Unlike the amino acid residues of the *N*-terminal domain implicated in zinc binding and action at the GlyR, amino acids important for α 1 GlyR modulation by alcohols and anesthetics are located in transmembrane domains two and three (TM2 and TM3). Specifically, serine-267 and alanine-288 play a role in GlyR enhancement by alcohols and anesthetics (Mihic et al., 1997), although residues in TM1 and TM4 may also contribute (Lobo et al., 2004, 2006, 2008). Cysteine substitution experiments at S267 and A288, involving covalent thiol binding or cross-linking, suggest an alcohol and anesthetic binding pocket among the transmembrane domains (Mascia et al., 2000; Lobo et al. 2006, 2008). Additional putative alcohol binding sites on the α 1 GlyR have also been reported. These include alanine-52, which is in Loop 2 of the *N*-terminal domain (Davies et al., 2004; Crawford et al., 2007), as well as lysine-385 (K385) of the large intracellular loop linking TM3 and TM4 (Yvenes et al., 2008). The latter residue has also been suggested to be involved in GlyR modulation by G β γ (Yvenes et al., 2003).

We hypothesize that the zinc enhancement of ethanol action at the GlyR may occur as a result of an interaction that increases the affinity of receptors for glycine. Single channel electrophysiological studies of zinc effects on the GlyR show many similarities to how ethanol exerts its effects. Both enhance the durations of bursts of channel opening activity, with increased numbers of openings seen per burst. Neither modulator affects mean channel open times and neither affects conductance (Laube et al., 2000; Welsh et al., 2009). In both studies it was concluded that the modulators act primarily by antagonizing glycine unbinding. If zinc and ethanol have these similar actions when each is applied on its own it is reasonable to hypothesize that an interaction between the two might also occur via this mechanism.

Recent behavioral studies in rodents have revealed possible effects of zinc on ethanol consumption, as well as a role for GlyRs in ethanol consumption. For example, Jones et al. (2008) suggest a significant correlation between hippocampal zinc levels and voluntary alcohol drinking. Female recombinant inbred BXD mice with relatively lower concentrations of zinc in hippocampus showed higher alcohol acceptance in a two-bottle choice drinking test. In addition, recent evidence implies that GlyRs in the nucleus accumbens may play a role in the voluntary consumption of ethanol. Microdialysis of glycine into the nucleus accumbens increased extracellular accumbal dopamine levels, and this was accompanied by a decrease in alcohol consumption by alcohol-preferring Wistar rats; in contrast, strychnine had the opposite effects (Molander et al., 2005). Strychnine, applied via microdialysis, also prevented increases of accumbal dopamine levels after either local or systemic alcohol administration (Molander and Söderpalm, 2005). In line with these findings, the glycine reuptake inhibitor, Org 25935, decreased EtOH, but not water, intake as well as EtOH preference (Molander et al., 2007).

In conclusion, low concentrations of zinc combined with ethanol produce increases in the magnitude of ethanol's effects on wild-type $\alpha 1$ GlyR function. However, low concentrations of zinc combined with isoflurane or pentanol do not affect the magnitude of $\alpha 1$ GlyR function enhancement by these modulators. We hypothesize that the co-application of zinc with either pentanol or isoflurane produces additive effects on GlyR function because the relatively higher efficacies of these compounds, compared to ethanol, allows lower concentrations be used, consequently leading to a lower probability of any given GlyR binding both zinc and either pentanol or isoflurane simultaneously. In addition, we posit that zinc and ethanol interact at $\alpha 1$ GlyRs to increase the magnitude of the effects of each individual compound. We hypothesize that this zinc/ethanol interaction at the GlyR may occur as a result of the two compounds acting together to increasing glycine affinity to a greater degree than when each is present in isolation. Finally, our findings raise the possibility that ethanol effects on GlyR function observed *in vitro* may not estimate its true effects *in vivo* and that endogenous compounds such as zinc may play a key role in determining the magnitude ethanol effects *in vivo*.

Acknowledgments

This work was supported by NIH grants R01AA11525, R01AA06399 and P01GM47818.

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Figure 1.

Currents elicited by glycine were determined before (solid symbols) and after (hollow symbols) application of 10 mM tricine. Tricine did not affect the currents elicited by a maximally-effective concentration of glycine (10 mM) although it did increase the glycine EC_{50} from 127 μ M to 219 μ M; i.e., it only decreased the effects of submaximal glycine concentrations. The Hill coefficient increased from 1.9 in the absence of tricine to 3 in its presence. Data represent the mean $\pm$ S.E.M. from 3 oocytes.

Figure 2.

GlyR modulation by ethanol and zinc were determined before and after application of 10 mM tricine. A) 50 mM ethanol enhancement of sub-maximal glycine responses was determined first in the absence and then in the presence of 10 mM tricine. The glycine concentration corresponding to EC5-10 was calculated in the absence of tricine, and then that same sub-maximal glycine concentration was subsequently used to determine the effects of tricine on GlyR modulation by ethanol and zinc. Glycine was applied for 30 sec. When ethanol was tested it was pre-applied to the oocyte for 60 sec before also being applied with glycine. B) Tricine decreases the degree of 20-200 mM ethanol enhancement observed. Currents produced by EtOH plus EC5-10 glycine were compared to EC5-10 glycine, both in the absence (solid bars)

and presence (hollow bars) of tricine. C) Tricine completely prevents the ability of 1 μ M zinc to enhance sub-maximal glycine responses. Currents produced by zinc plus EC5-10 glycine were compared to EC5-10 glycine, both in the absence (solid bars) and presence (hollow bars) of tricine. Data represent the mean $\pm$ S.E.M. from 4-5 oocytes.

Figure 3.

A) 200 mM ethanol was first co-applied with 10 μ M glycine and then was reapplied after a washout period with both 10 μ M glycine and 50 nM zinc. B) The amount of current produced by 200 mM ethanol was significantly greater when it was applied during a concurrent application of 50 nM zinc + 10 μ M glycine than when it was applied with 10 μ M glycine alone. Data represent the mean $\pm$ S.E.M. from 11 oocytes.

Figure 4.

A) 0.6 mM isoflurane was applied in the absence of and in the presence of 50 nM zinc as described in Figure 3. A & B) The magnitude of the current produced by 0.6 mM isoflurane or 2.9 mM pentanol, in the presence of 10 μ M glycine, was the same regardless of whether 50 nM zinc was present or not. Data represent the mean $\pm$ S.E.M. from 3-4 oocytes.