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Zinc Is a Mixed Antagonist of Homomeric ρ1 γ-Aminobutyric Acid-Activated Channels

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SUMMARY

The transition metal Zn^{2+} is differentially distributed in the central nervous system, where it is proposed to be a neuromodulator. One of the documented effects of Zn^{2+} is the antagonism of γ-aminobutyric acid (GABA)-mediated synaptic inhibition. This antagonism is presumed to result from a direct interaction of Zn^{2+} with the GABA receptor/ionophore complex, although the characteristics of Zn^{2+} sensitivity are dependent on the particular GABA subunit combination. In this study, we examined the effects of Zn^{2+} on homomeric ρ1 GABA-activated channels expressed in *Xenopus* oocytes. Zn^{2+} was found to be a mixed antagonist of these recombinant ρ1 GABA receptors. The antagonism was predominately competitive at low Zn^{2+} concentrations (≤100 μM), whereas at high Zn^{2+} concentrations (>100 μM) a noncompetitive antagonism was apparent. Evidence is presented showing that the antagonism was not due to an interaction of GABA and Zn^{2+} in solution but, rather, resulted from interactions of these two ligands with the GABA-activated channel. A mechanism is proposed for Zn^{2+}-mediated antagonism in which GABA and Zn^{2+} bind to distinct sites on the GABA complex. The apparent mixed antagonism may arise from different K_i values for the binding of Zn^{2+} to non-agonist-bound or agonist-bound receptors. However, two distinct Zn^{2+} binding sites, one competitive and one noncompetitive, could also give rise to the dual antagonism.

The release of GABA from presynaptic terminals in the central nervous system produces a postsynaptic chloride flux that inhibits the neuron. This chloride flux is mediated by a membrane protein consisting of a receptor for the GABA molecule linked to a chloride-selective ion pore. The vital role of GABA-mediated inhibition in brain function has motivated detailed investigations of the properties of these GABA-gated chloride channels.

Molecular cloning studies have revealed several different classes of GABA receptor subunits (α, β, γ, δ, and ρ), with multiple isoforms in each class (1–4). It is presumed, by analogy with other members of this receptor-operated ion channel superfamily (e.g., glycine-activated and nicotinic acetylcholine-activated channels), that the GABA-activated channel is composed of five subunits (5, 6). The ρ1 GABA receptor subunit is predominately expressed in the retina (7) and forms functional homomeric GABA receptors when expressed in *Xenopus* oocytes (7). These homomeric channels have very different properties than do typical heteromeric GABA receptors (e.g., αβγ). For example, the relative efficacy of GABA agonists differs between the two types of GABA receptors (8). In addition, the activation and deactivation kinetics of the ρ1 receptor are much slower than those of typical heteromeric GABA receptors (9). Finally, ρ1 GABA-activated currents are not modulated by barbiturates and benzodiazepines, nor are they blocked by bicuculline (10). Effects of these compounds are considered hallmarks of the $GABA_A$ receptor, prompting the designation of a new class of GABA receptors, $GABA_C$ (11). Some retinal cell types also demonstrate $GABA_C$-type responses (12–15), suggesting that their GABA receptors may be composed of ρ subunits.

The transition metal Zn^{2+} is differentially distributed in the central nervous system (16) and has been shown to alter synaptic transmission (17–19). One of the documented actions of Zn^{2+} is antagonism of GABA-mediated inhibition, presumably through a direct interaction of Zn^{2+} with the GABA receptor/ionophore complex. The effects of Zn^{2+}, however, depend on the particular GABA subunit combination. For example, αβ subunits are potently antagonized by Zn^{2+}, whereas αβγ GABA receptors are relatively Zn^{2+} resistant, indicating that the γ subunit confers this Zn^{2+} resistance (20, 21). Concerning the mechanism of antagonism, Zn^{2+} noncompetitively inhibits GABA-activated currents from neurons of the rat superior cervical ganglion (22) and hippocampus (17, 23, 24) and from recombinant GABA receptors composed of α and β subunits (20, 21). Alternatively, there are reports of Zn^{2+} inhibition of GABA-activated currents in spinal cord neurons (25) and dorsal root ganglion cells (26) that is surmountable by GABA, suggesting the presence of a

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ABBREVIATIONS: GABA, γ-aminobutyric acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

competitive antagonism. Zn^{2+} is found in the retina, and electrophysiological experiments have shown that Zn^{2+} reduces GABA-mediated depolarizations in horizontal cells (27), although the mechanism of this antagonism has not been elucidated.

In this study, the effects of Zn^{2+} on GABA-activated channels composed of ρ1 subunits expressed in *Xenopus* oocytes were examined. Zn^{2+} blocked these GABA-activated currents in an apparently mixed fashion. At low Zn^{2+} concentrations ($\leq$100 μM), the antagonism was totally surmountable by GABA, suggesting competitive inhibition. At higher Zn^{2+} concentrations (>100 μM), a noncompetitive component was apparent. A model is presented in which Zn^{2+} binds to an allosteric site on the protein distinct from the GABA recognition site. In this model, GABA and Zn^{2+} can be bound simultaneously to the channel complex. The apparent mixed antagonism arises from different K_i values for the binding of Zn^{2+} to either non-GABA-bound or GABA-bound receptors. However, there could also be two distinct Zn^{2+} binding sites, one competitive and one noncompetitive, giving rise to the dual antagonism.

Materials and Methods

Isolation of cDNAs, site-directed mutagenesis, and *in vitro* transcription. Polymerase chain reaction protocols used to isolate the cDNAs encoding the α1, β2, γ2, and ρ1 subunits of the GABA receptor have been reported previously (9, 28). Procedures for construction of the mutant subunits used in this study have also been described previously (9). Before the cRNA was made, the mutant and wild-type cDNAs were linearized with *Nhe*I (ρ1 subunit) or *Ssp*I (α1, β2, and γ2 subunits). cRNA was transcribed from the linearized cDNAs using Megascript (Ambion, Austin TX).

Expression of GABA receptors in *Xenopus* oocytes. Female *Xenopus laevis* were anesthetized by hypothermia, and ovarian lobes were removed from the frog and placed in a sterile OR2 solution consisting of 82.5 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 10 mM Na_2HPO_4, 50 units/ml penicillin, and 50 μg/ml streptomycin, pH 7.5. The ovarian lobes were cut into small pieces and placed in a slow-stir vessel with Ca^{2+}-free OR2 and 0.3% collagenase A (Boehringer Mannheim, Indianapolis, IN) for 1.5–2 hr. The dispersed oocytes were then thoroughly rinsed with OR2. Defolliculated, stage VI oocytes were selected under the microscope and maintained at 18° for several hours or overnight before injection of cRNA.

Micropipettes for injecting cRNA were fabricated with a P-87 horizontal micropipette puller (Sutter Instruments, Novato, CA), and the tips were cut with scissors under the microscope. For experiments involving the coexpression of α1, β2, and γ2 subunits, the cRNAs were mixed at a ratio of 1:1:4, to increase the likelihood that the expressed channels contained a γ2 subunit. The cRNA was then diluted with diethylpyrocarbonate-treated water, drawn up into the micropipette, and injected into oocytes using a Drummond Nanoject injector (Drummond Scientific Co., Broomall, PA).

Voltage clamp. One to 2 days after injection, the oocytes were placed on a 300-μm nylon mesh suspended in a small-volume chamber (<100 μl). The chamber has an inlet in the top and an outlet in the bottom, allowing continuous perfusion of the oocyte. Eighteen separate 60-ml syringe barrels were used to contain the perfusion solutions. A stock solution of GABA in OR2 was made fresh each day.

Recording microelectrodes were fabricated on a horizontal micropipette puller (Sutter P-87) and filled with 3 M KCl. The resistances of the electrodes were typically 1–3 MΩ. The two-electrode voltage-clamp technique was used to examine GABA-activated chloride currents in the oocytes. In all cases, the membrane potential was clamped at −70 mV. The data were recorded with a Gould chart recorder, as well as being recorded with a videocassette recorder for off-line analysis.

Data analysis. Because of the variability of expression levels between oocytes, data were normalized to the maximum current for each oocyte. Reported estimates of the mean and standard error were determined from the normalized data.

To quantify the inhibitory effect of Zn^{2+} at a fixed GABA concentration, plots of Zn^{2+} concentration versus normalized current amplitude were fitted with the following multisite inhibition equation, using a nonlinear least-squares method:

$$I = \frac{I_{max}}{1 + ([Zn^{2+}]/IC_{50})^n} \quad (1)$$

In eq.1, I is the peak or steady-state current for a given Zn^{2+} concentration, I_{max} is the current amplitude in the absence of Zn^{2+} (equal to 1, because the data were normalized to the maximum), IC_{50} is the concentration of Zn^{2+} required to block half of the GABA-activated current, and n is the slope factor. Dose-response relationships for GABA in the absence and presence of a fixed concentration of Zn^{2+} were fit with the following logistic equation:

$$I = \frac{I_{max}}{1 + (EC_{50}/[GABA])^n} \quad (2)$$

where EC_{50} is the concentration of GABA required for a half-maximal response. At high Zn^{2+} concentrations the GABA-activated current declined to a steady-state level. The amplitude of this current

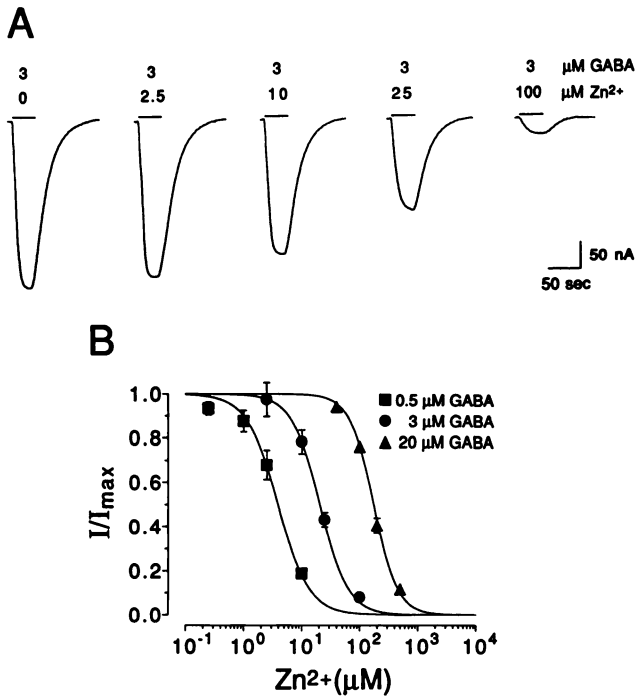

Fig. 1. GABA-activated currents from ρ1 GABA receptors were competitively antagonized by Zn^{2+}. A, *Xenopus* oocytes expressing ρ1 GABA-activated channel subunits were two-electrode voltage-clamped at −70 mV. Currents were examined in response to 3 μM GABA in the presence of 0, 2.5, 10, 25, and 100 μM Zn^{2+}. Zn^{2+} reduced the current amplitude in a concentration-dependent manner. B, The effects of Zn^{2+} on currents activated by different concentrations of GABA were examined as in A. Each *point* is the mean ± standard error from measurements of the peak of the GABA-activated current in five or six oocytes. *Continuous lines*, best fits of a multisite inhibition equation (see Materials and Methods) to the data points. The parameters from these fits are presented in Table 1. Increasing the GABA concentration shifted the Zn^{2+} inhibition curve to the right.

plateau was determined either by visual inspection or by fitting an exponential component plus offset to the latter part of the current decay. If the application of Zn^{2+} was maintained for sufficiently long time periods, estimates from these two methods yielded values within 5% of one another. All data acquisition and analysis software was written in the laboratory for the Macintosh computer (Apple, Cupertino CA).

Results

Zn^{2+} Inhibits GABA-Activated Currents from Homomeric ρ1 GABA Receptors

Xenopus oocytes were injected with cRNA encoding the ρ1 GABA receptor subunit isolated from a human retinal cDNA library (7, 9). One to 3 days later the oocytes were two-electrode voltage-clamped and GABA-activated currents were measured in the presence of increasing concentrations of Zn^{2+}. Fig. 1A shows currents activated by 3 μM GABA alone and in the presence of 2.5, 10, 25, and 100 μM Zn^{2+}. Note that Zn^{2+} reduced the peak amplitude of the current in a concentration-dependent manner. Fig. 1B shows a plot of the relationship between the peak of the GABA (3 μM)-activated currents (normalized to the maximum) and the Zn^{2+} concentration. The data points represent the mean and standard error of five experiments. A multisite inhibition model was fit to these data and yielded an IC_{50} (concentration of Zn^{2+} required to block half of the current) of 20.4 ± 2.6 μM and a slope factor of 1.77 ± 0.30 (Table 1).

Zn^{2+} Inhibition Appears Competitive at Low Concentrations

If the inhibition is competitive, then increasing the GABA concentration should reduce the Zn^{2+} antagonism. To test this hypothesis, we examined Zn^{2+} antagonism of currents activated by different concentrations of GABA. The first indication that the antagonism by Zn^{2+} was, at least partly, competitive is presented in Fig. 1B, which shows Zn^{2+} antagonism of currents activated by 0.5, 3, and 20 μM GABA (in experiments similar to those in Fig. 1A). Increasing the concentration of GABA shifted the Zn^{2+} inhibition curve to the right (IC_{50} values are presented in Table 1).

To investigate whether GABA can totally overcome the Zn^{2+} block, we examined dose-response relationships for GABA in the presence of several different Zn^{2+} concentrations. The closed symbols in Fig. 2 are GABA dose-response relationships in the presence of 0, 10, and 100 μM Zn^{2+}. Increasing the concentration of Zn^{2+} induced a rightward shift in the dose-response relationship for GABA. The continuous lines are the best fits of the logistic equation to these data. The GABA EC_{50} (concentration of GABA required for half-maximal current) in the presence of 0, 10, and 100 μM Zn^{2+} was 0.83 ± 0.04, 1.77 ± 0.09, and 12.62 ± 1.23 μM (n = 4), respectively. The inhibition by Zn^{2+}, even at 100 μM, was totally surmounted by increasing GABA concentrations. Note the slight enhancement of the maximum GABA-activated current evident in the presence of 10 and 100 μM Zn^{2+}. This enhancement was not due to time-dependent changes in the oocyte, because it was still observed when the experimental acquisition sequence was reversed (i.e., 100 μM Zn^{2+}, 10 μM Zn^{2+}, and then 0 μM Zn^{2+}). The data presented in Fig. 2 demonstrate that increasing the GABA concentration can completely overcome the Zn^{2+} inhibition (for Zn^{2+} concentrations of ≤100 μM), further indicating a competitive mechanism of antagonism.

Interactions of Zn^{2+} with amino acids and proteins are well documented (29–31) and raise the possibility that the competitive antagonism of GABA-activated currents presented in Figs. 1 and 2 may be due to the formation of an inactive GABA-Zn^{2+} complex. In the next section we present three lines of evidence that the competition between GABA and Zn^{2+} is not in solution but, rather, results from the interactions of Zn^{2+} and GABA with the GABA receptor complex.

The Competitive Antagonism Is Not Due to a Direct Interaction between GABA and Zn^{2+} in Solution

Zn^{2+} antagonizes αβγ GABA receptors. αβγ GABA receptors have been reported to be Zn^{2+} insensitive at low Zn^{2+} concentrations (20, 21). This Zn^{2+} insensitivity is pre-

TABLE 1

Parameters determined from fitting a multisite inhibition equation to dose (Zn^{2+})-response relationships, as described in Materials and Methods

All IC_{50} and n values are mean ± standard error. The EC_{50} values are from the work of Amin and Weiss (9, 32).

Subunit	EC_{50}	[GABA]	[GABA]/EC_{50}	Zn^{2+} IC_{50}	n	No. of oocytes
	μM	μM		μM		
Wild-type ρ1	1.16	0.5	0.43	3.89 ± 0.54	1.61 ± 0.22	5
		3	2.59	20.42 ± 2.55	1.77 ± 0.30	5
		20	17.24	172.92 ± 9.50	2.09 ± 0.13	6
Y198F ρ1	13.2	5	0.38	3.11 ± 0.88	1.11 ± 0.01	3
		30	2.27	38.23 ± 11.61	1.48 ± 0.21	3
		180	13.64	330.48 ± 114.60	1.70 ± 0.06	3
Y198S ρ1	2910	1,000	0.34	8.36 ± 0.23	1.39 ± 0.03	7
		3,000	1.03	20.50 ± 1.02	1.22 ± 0.03	7
		4,500	1.55	32.27 ± 4.06	1.41 ± 0.06	5
		10,000	3.44	69.92 ± 2.48	1.28 ± 0.02	7
Y247F ρ1	55.1	15	0.27	2.84 ± 0.19	1.24 ± 0.05	7
		30	0.54	6.94 ± 0.80	1.25 ± 0.02	4
		75	1.36	14.29 ± 0.89	1.32 ± 0.03	7
		180	3.27	51.84 ± 5.58	1.40 ± 0.03	4
		400	7.26	109.56 ± 9.56	1.33 ± 0.05	7
α1β2γ2	45.8	3	0.07	441.33 ± 39.79	1.22 ± 0.05	4
		20	0.44	648.40 ± 63.11	1.23 ± 0.04	4
		100	2.18	2,927.15 ± 493.14	1.04 ± 0.03	3

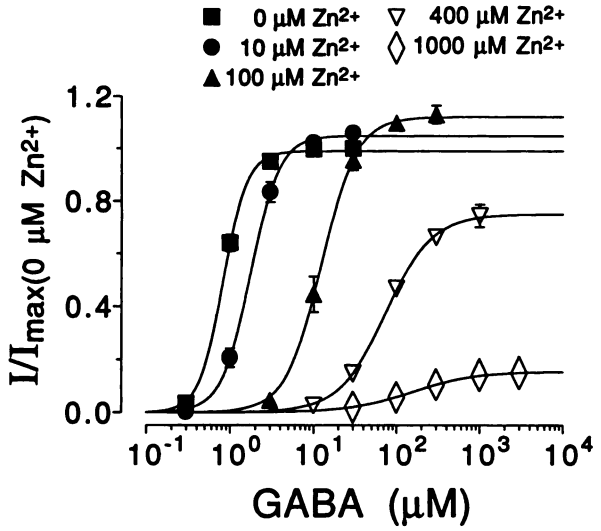

Fig. 2. Zn^{2+} antagonism is surmountable by GABA at low concentrations of Zn^{2+}, whereas at high Zn^{2+}-concentrations the antagonism is not totally surmountable by GABA. The peak amplitudes of GABA-activated currents were measured in response to different concentrations of GABA in the presence of fixed Zn^{2+} concentrations (*closed symbols*). The data were normalized to the maximum amplitude in the absence of Zn^{2+} for each individual oocyte. Increasing the concentration of Zn^{2+} shifted the GABA dose-response relationship to the right. *Continuous lines*, best fits of the logistic equation to the data points. The GABA EC_{50} values with 0, 10, and 100 μM Zn^{2+} were 0.83 ± 0.04, 1.77 ± 0.09, and 12.62 ± 1.23 μM, respectively (four oocytes). At these lower Zn^{2+} concentrations, the maximum obtainable current was not decreased by Zn^{2+}, indicating a competitive inhibition. Note the slight enhancement of the maximum with 10 and 100 μM Zn^{2+}. The dose-response relationship obtained when the current plateau, seen at higher Zn^{2+} concentrations, was measured is also shown (*open symbols*). The GABA EC_{50} values in the presence of 400 and 1000 μM Zn^{2+} were 75.1 ± 3.9 and 162.5 ± 15.5 μM, respectively (six oocytes). In these cases, the maximum current was not surmountable by GABA, suggesting a noncompetitive mechanism of inhibition. The IC_{50} for this noncompetitive antagonism was estimated to be ~550 μM, with a slope factor of ~3.0. These values were determined by fitting eq. 1 to the relationship between Zn^{2+} concentration and the predicted, normalized, maximum current at each Zn^{2+} concentration.

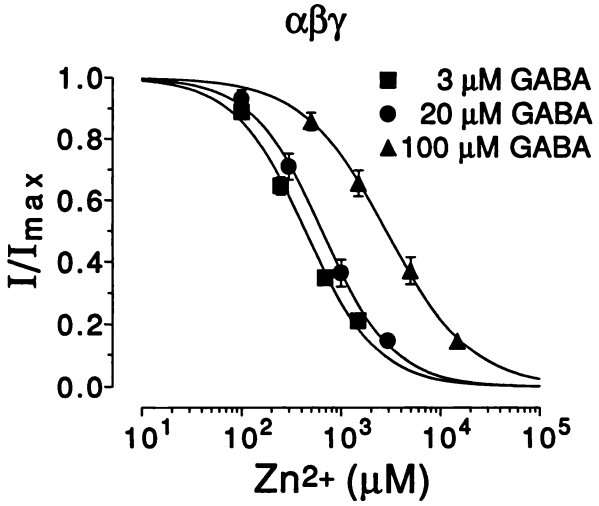

Fig. 3. Higher concentrations of Zn^{2+} were required to antagonize GABA-activated channels composed of $\alpha1\beta2\gamma2$ subunits, compared with $\rho1$ GABA-activated channels. Oocytes were injected with cRNA encoding $\alpha1$, $\beta2$, and $\gamma2$ subunits, at a ratio of 1:1:4. This ratio was used to increase the likelihood that the expressed GABA-activated channels contained a $\gamma2$ subunit. The $\alpha1\beta2\gamma2$ currents were competitively antagonized by Zn^{2+} (parameters are given in Table 1), although the Zn^{2+} concentration required to block the channels was much higher than that required to block the $\rho1$ GABA-activated channel. These data argue against an interaction between GABA and Zn^{2+} in solution underlying the observed competitive antagonism of the $\rho1$ receptor.

sumably conferred by the presence of the γ subunit. If an interaction of Zn^{2+} and GABA in solution lowers the free GABA concentration, thereby accounting for the competitive antagonism of $\rho1$ GABA receptors, then a similar competitive antagonism (similar IC_{50} value) should be observed for Zn^{2+} with $\alpha\beta\gamma$ GABA receptors. The data in Fig. 3 demonstrate that, although Zn^{2+} does competitively antagonize $\alpha\beta\gamma$ GABA receptors, this antagonism requires much higher Zn^{2+} concentrations than that required to antagonize the $\rho1$ receptor (Table 1). For example at comparable GABA concentrations (with respect to their GABA EC_{50} values), the IC_{50} for Zn^{2+} was 3.9 ± 0.54 μM for the $\rho1$ GABA receptor and 648 ± 63 μM for the $\alpha\beta\gamma$ receptor. These data argue against an interaction between GABA and Zn^{2+} in solution underlying the competitive antagonism of the $\rho1$ GABA receptor.

Mutations that impair GABA sensitivity do not affect competitive antagonism by Zn^{2+}. Amino acids have been identified in the $\rho1$ subunit that appear to be either part of, or very near, the GABA binding site (9, 32). These amino acids exist in two separate domains of the amino-terminal extracellular region. Mutation of these amino acids dramatically increased the concentration of GABA required to activate the channel. If the competitive antagonism was due to the formation of an inactive GABA-Zn^{2+} complex, then the Zn^{2+} concentration required for channel block should increase along with the concentration of GABA required to activate these mutant receptors.

The GABA EC_{50} for the Y198F mutant (tyrosine at position 198 mutated to phenylalanine) was 13.2 μM, compared with the wild-type EC_{50} of 1.16 μM (9). This represents an 11.4-fold reduction in GABA sensitivity. Fig. 4A shows the effects of Zn^{2+} on Y198F mutant channel currents activated by 5 μM, 30 μM, and 180 μM GABA. In terms of the EC_{50}, these GABA concentrations are comparable to 0.5 μM, 3.0 μM, and 20 μM GABA for the wild-type receptor (Fig. 1B). Table 1 presents the IC_{50} values and slope factors for fits of a multisite inhibition function to the data points. Table 1 also presents the ratio of the GABA concentration to the EC_{50}, to facilitate direct comparison between the wild-type and mutant receptor Zn^{2+}-inhibition parameters. Although the Y198F mutant channel required an ≈11-fold higher GABA concentration for activation, the IC_{50} for Zn^{2+} (at comparable GABA concentrations, with respect to the EC_{50}) was similar to that of the wild-type receptor (Table 1).

Substitution of the tyrosine at position 198 with a serine (rather than a phenylalanine as for the mutant in Fig. 4A) produced GABA-activated channels that were ≈2500-fold less sensitive to GABA (9). Fig. 4B shows Zn^{2+} inhibition of currents activated by four different concentrations of GABA with the Y198S mutant. Due to the dramatic reduction in GABA sensitivity with this particular mutant, it was not possible to examine as broad a GABA concentration range as for the wild-type receptor and the Y198F mutant (osmotic effects occur with very high agonist concentrations). Nevertheless, the competitive inhibition was still prevalent and the Zn^{2+} IC_{50}, at comparably effective GABA concentrations, was similar to that of the wild-type channel (Table 1). Fi-

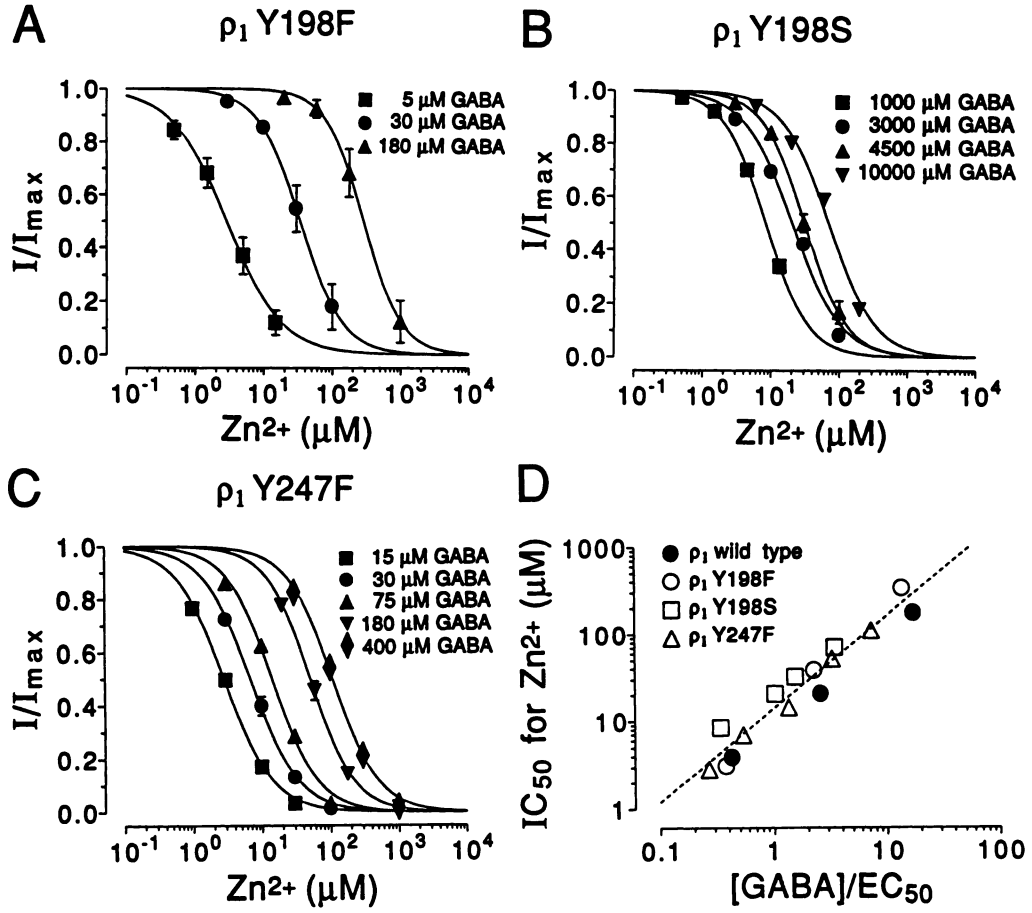

Fig. 4. Mutations that impaired GABA activation did not affect antagonism by Zn^{2+}. A, Zn^{2+} antagonism was examined for GABA-activated currents from the ρ1 subunit containing a mutation in the presumed agonist binding site, namely Y198F (tyrosine at position 198 mutated to phenylalanine). Although these GABA-activated channels were ~11-fold less sensitive to GABA, compared with the wild-type channel, similar concentrations of Zn^{2+} (compared with the wild-type channel) were required for block. The parameters determined by fitting a multisite inhibition equation to these data are presented in Table 1, along with the wild-type parameters. B, Zn^{2+} antagonism of the mutant Y198S receptor, which is ~2500-fold less sensitive to GABA than is the wild-type receptor, is shown. The Zn^{2+} concentration required to block the channels was similar to that required for the wild-type channel (Table 1). C, These data show Zn^{2+} antagonism of the mutant Y247F receptor, which is ~48-fold less sensitive to GABA. Again, the Zn^{2+} concentration required to block the mutant channels was similar to that needed for the wild-type receptor. D, A log-log plot of the IC_{50} versus the normalized (to the EC_{50}) GABA concentration for the wild-type ρ1 receptor and mutant ρ1 receptors is shown. *Dashed line*, linear regression for all points. Even though very different concentrations of GABA were required to activate the wild-type and mutant receptors, similar concentrations of Zn^{2+} were required to antagonize them.

nally, Fig. 4C shows Zn^{2+} inhibition of Y247F mutant channel currents activated by five different GABA concentrations. This mutation produced an ~48-fold increase in the GABA EC_{50} (9). As shown in Fig. 4C and Table 1, the concentration range of Zn^{2+} required to inhibit GABA-activated currents with the Y247F mutant receptor was comparable to that with the wild-type receptor.

The data in Fig. 4, A–C, are summarized in Fig. 4D, which is a plot of the GABA concentration (normalized to the EC_{50}) versus the Zn^{2+} IC_{50} for the wild-type, Y198F, Y198S, and Y247F receptors. It is clear from the data presented in Fig. 4D that, even when very high concentrations of GABA are required to activate the channels, similar concentrations of Zn^{2+} are required for block. These data argue against an interaction between Zn^{2+} and GABA in solution underlying the antagonism.

Zn^{2+} affects the decay of GABA-activated currents after GABA removal. GABA-activated currents from oocytes expressing homomeric ρ1 GABA-activated channels decay very slowly when agonist is removed (9). This deactivation rate is exemplified in the most slowly decaying current record of Fig. 5. This decay is not determined by the time required to remove agonist from the bath, which takes <3 sec (9). Thus, the decay rate is limited by channel closing and/or GABA dissociation from its receptor. The other, more quickly decaying, current traces in Fig. 5 show deactivation when the bath solution was switched to 0 μM GABA plus different concentrations of Zn^{2+}. The higher the concentration of Zn^{2+}, the faster the rate of decay. Even though GABA was removed from the bath within 3 sec, the Zn^{2+} concentration in the bath still determined the rate of decay. This argues against a Zn^{2+}-GABA interaction in solution underlying the antagonism.

Higher Concentrations of Zn^{2+} Noncompetitively Antagonize Homomeric ρ1 GABA-Activated Channels

Fig. 6 shows currents activated by 100 μM GABA along with 0, 100, 250, 700, or 2000 μM Zn^{2+}. Note that, in the presence of 250 μM Zn^{2+} (and higher), the GABA-activated currents reached a peak and then decayed to a steady state

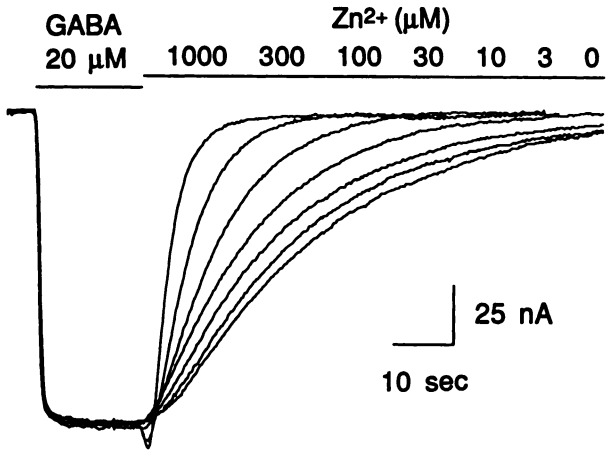

Fig. 5. Zn^{2+} increased the rate of decay of GABA-activated currents. Currents were first activated by 20 μM GABA. After the current reached steady state, GABA was washed out at the same time that Zn^{2+}, at the different concentrations indicated (at the *top*), was washed in. Increasing the concentration of Zn^{2+} increased the decay rate. Even though GABA was removed from the bath within 3 sec (9), the Zn^{2+} concentration increased the rate of decay. Similar results were obtained in five oocytes.

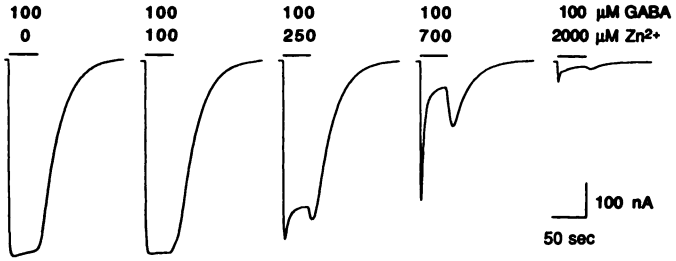

Fig. 6. Zn^{2+}, at high concentrations, noncompetitively antagonized GABA-activated currents. Currents were activated by 100 μM GABA in the absence and then in the presence of Zn^{2+} at the indicated concentrations. At 250 μM Zn^{2+} and above, the currents exhibited a peak followed by a rapid decay to a plateau. Upon Zn^{2+} and GABA washout, there was a current rebound due to the reopening of channels as Zn^{2+} left its binding site. Similar results were obtained in six oocytes.

level. There was also a partial rebound of the current when GABA and Zn^{2+} were removed from the bath. This rebound most likely resulted from the more rapid dissociation of Zn^{2+} than GABA from their respective binding sites, allowing reopening of the channel. Compare the current traces in Fig. 6 with those in Fig. 1A, where lower Zn^{2+} and GABA concentrations were examined. The lower concentrations of Zn^{2+} and GABA in Fig. 1A resulted in an immediate block; no significant decay from the peak was evident. The open symbols in Fig. 2 are GABA dose-response relationships in the presence of 400 and 1000 μM Zn^{2+}, which were determined by measuring the current plateau. At these higher Zn^{2+} concentrations, the inhibition, as measured from the plateau, could not be surmounted by increasing GABA concentrations. Thus, the data presented in Figs. 2 and 6 suggest that Zn^{2+}, at high concentrations, can noncompetitively antagonize ρ1 GABA-activated currents.

The decay in the current induced by high concentrations of Zn^{2+} (Fig. 6) might be interpreted as a use-dependent block, suggesting that the channels must open before the block can occur. That this is not the case is shown in Fig. 7. Fig. 7, *left*, shows a response to the simultaneous application of 100 μM

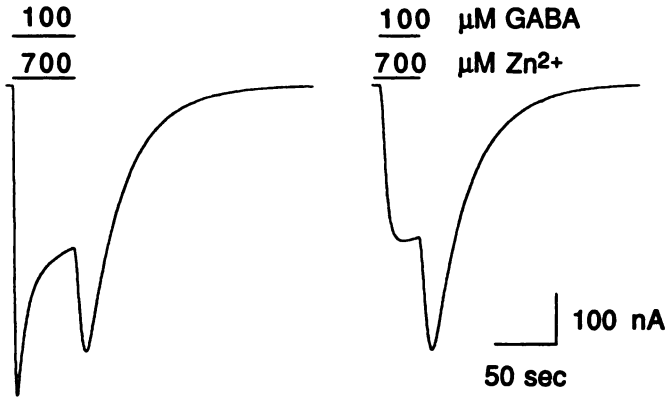

Fig. 7. Preincubation with Zn^{2+} eliminated the peak and current decay. *Left*, 100 μM GABA and 700 μM Zn^{2+} were coapplied. The current decayed towards a plateau and then rebounded upon GABA and Zn^{2+} removal. *Right*, the oocytes were preincubated with Zn^{2+} for 4 sec before the application of GABA. This preincubation eliminated the peak and decay, and the current rose directly to the plateau level. This indicates that Zn^{2+} can bind to the channel before activation by GABA. Similar results were obtained in four oocytes.

GABA and 700 μM Zn^{2+}. The current decayed from the peak towards a plateau and rebounded upon washout. In Fig. 7, *right*, 700 μM Zn^{2+} was applied for ≈4 sec before the 100 μM GABA/700 μM Zn^{2+} coapplication. The initial peak was nearly abolished. The conclusion from this experiment is that Zn^{2+} can bind to and inhibit the channel before the binding of GABA. The initial peak evident in the current traces of Figs. 6 and 7 is a result of the faster rate of activation by GABA, compared with the slower rate of block by Zn^{2+}. This is considered further in Discussion.

The current traces in Fig. 8 illustrate and summarize the effects of Zn^{2+} that we have characterized. In this experiment, GABA was first applied at the indicated concentration. After the current reached a steady state, Zn^{2+} was coapplied for several seconds and then removed. All three current traces in Fig. 8 show the same ratio of GABA to Zn^{2+} con-

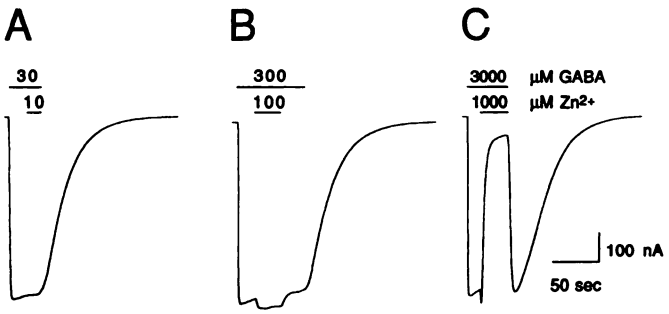

Fig. 8. The amplitude of the GABA-activated current is not solely determined by the ratio of the GABA and Zn^{2+} concentrations. In all three traces, currents were activated by the indicated concentration of GABA. After a steady state was obtained, Zn^{2+} (at the indicated concentration) was introduced for several seconds and then removed. In all three experiments the ratio of the Zn^{2+} concentration to the GABA concentration was the same (3:1). A, GABA at 30 μM surmounted the antagonism by 10 μM Zn^{2+}. B, GABA at 300 μM surmounted the antagonism by 100 μM Zn^{2+}, and a slight enhancement of the current was prevalent. C, GABA at 3000 μM could not overcome the inhibitory effects of 1000 μM Zn^{2+}, even though the ratio of GABA to Zn^{2+} was the same as that in A and B. Similar results were obtained in four oocytes.

centration, namely 3:1. In Fig. 8A, with 30 μM GABA and 10 μM Zn^{2+} no block was observed, because this concentration of GABA surmounted the Zn^{2+} antagonism (Fig. 1B). In Fig. 8B, with 300 μM GABA and 100 μM Zn^{2+} the competitive inhibition by Zn^{2+} was surmounted by GABA and the slight enhancement of the current was evident. In Fig. 8C, with 3000 μM GABA and 1000 μM Zn^{2+} the noncompetitive antagonism was observed and the current was substantially blocked. The data in Fig. 8 demonstrate that the degree of inhibition is not simply determined by the ratio of agonist to antagonist concentrations.

Discussion

This study examined the effects of Zn^{2+} on GABA-activated currents from homomeric ρ1 GABA receptors expressed in *Xenopus* oocytes. Zn^{2+} appeared to be a mixed antagonist of homomeric ρ1 GABA-activated channels. At a lower Zn^{2+} concentration range (0.25–100 μM), the antagonism appeared predominately competitive. This is based on the observations that 1) increasing the concentration of GABA increased the IC_{50} for Zn^{2+} (Fig. 1B) and 2) Zn^{2+} shifted the GABA dose-response relationship to higher GABA concentrations (Fig. 2). Because the maximum GABA-activated current in the presence of low Zn^{2+} concentrations could be completely recovered by increasing the GABA concentration, the inhibition was predominately competitive.

Interactions of Zn^{2+} with amino acids and proteins are well documented (29–31) and raised the possibility that the competitive antagonism of GABA-activated currents may have been due to the formation of an inactive GABA-Zn^{2+} complex. Three lines of evidence indicated that this apparent competitive antagonism was not due to an interaction between GABA and Zn^{2+} in solution. 1) αβγ GABA-activated channels did not show competitive antagonism at Zn^{2+} and GABA concentrations similar to those used for the ρ1 studies (Fig. 3). (αβγ GABA-activated channels were blocked by much higher concentrations of Zn^{2+}, however.) 2) Mutant ρ1 GABA-activated channels that required much higher concentrations of GABA for activation were antagonized by similar concentrations of Zn^{2+} as was the wild-type ρ1 GABA-activated channel (Fig. 4). 3) The concentration of Zn^{2+} in the bath could determine the rate of GABA-activated current decay in the absence of GABA (Fig. 5). Thus, the competitive antagonism most likely resulted from interactions of Zn^{2+} with the GABA receptor/ionophore complex.

At higher Zn^{2+} concentrations (>100 μM), the GABA-activated current peaked and then decayed to a plateau. The relationship between GABA concentration and the amplitude of the current plateau at these higher Zn^{2+} concentrations indicated a noncompetitive mechanism of antagonism (maximum current was depressed). Thus, low Zn^{2+} concentrations were competitive and shifted the GABA EC_{50} to the right. High Zn^{2+} concentrations produced a mixed inhibition, in that there was a rightward shift in the EC_{50} as well as a depression in the maximum. Finally, there was a slight potentiation of the GABA-activated current that was evident in the presence of 10 and 100 μM Zn^{2+} (Figs. 2 and 8B). This potentiation may indicate an additional site of action of Zn^{2+} on the GABA receptor.

The possible mechanism for antagonism by Zn^{2+} will be considered in terms of the following simple kinetic model for activation of the GABA receptor:

$$A + R \overset{K_{d,R}}{\leftrightarrow} AR \leftrightarrow AR^* \qquad \text{(I)}$$

where A is the agonist molecule (GABA), $K_{d,R}$ is the dissociation constant for the binding of GABA to the receptor complex (R), AR is the agonist-bound closed channel, and AR^* is the agonist-bound open channel. Based on the Hill coefficient from GABA dose-response relationships, we know that this model is an oversimplification, because multiple agonist molecules must bind to activate the channel (9). Scheme I collapses the multiple agonist binding steps into one step and provides a simpler framework for considering the mechanism of Zn^{2+} antagonism.

Zn^{2+} can bind to the non-GABA-bound receptor, as indicated in scheme II.

$$\begin{array}{ccccc} & & Zn^{2+} & & \\ & & + & & \\ & & & \overset{K_{d,R}}{} & \\ A + R & & & \leftrightarrow AR \leftrightarrow AR^* & \qquad \text{(II)} \\ \overset{K_{i,R}}{\updownarrow} & & & & \\ A + R \cdot Zn^{2+} & & & & \end{array}$$

In scheme II, $K_{i,R}$ is the dissociation constant for the binding of Zn^{2+} to R, and $R \cdot Zn^{2+}$ is the Zn^{2+}-bound, non-GABA-bound receptor. Scheme II is supported by the apparent competitive (agonist-surmountable) antagonism seen at lower Zn^{2+} concentrations (Figs. 1 and 2). The generally accepted interpretation of a competitive antagonism between two ligands is that they have overlapping binding sites and their binding is therefore mutually exclusive. However, this need not be the case. For example, Zn^{2+} may bind at a different site than GABA and, through an allosteric mechanism, prevent the binding of GABA to its receptor (21, 22, 24, 25). Alternatively, the binding of Zn^{2+} may impede the access of GABA to its receptor. Evidence was presented that the GABA and Zn^{2+} binding sites are not superimposed, because mutations that impaired GABA activation did not impair Zn^{2+} antagonism (Fig. 4). A mutational analysis of GABA activation and Zn^{2+} antagonism may provide additional information on the spatial relationship between GABA and Zn^{2+} binding domains.

At high concentrations, Zn^{2+} noncompetitively antagonized the channel, as demonstrated in Figs. 2 and 6. This requires a modification of scheme II.

$$\begin{array}{ccccccc} Zn^{2+} & & & & Zn^{2+} & & \\ + & & \overset{K_{d,R}}{} & & + & & \\ A + R & & \leftrightarrow & & AR & \overset{k_o}{\leftrightarrow} & AR^* \\ \overset{K_{i,R}}{\updownarrow} & & & & \overset{K_{i,AR}}{\updownarrow} & \underset{k_c}{} & \\ A + R \cdot Zn^{2+} & & \leftrightarrow & & AR \cdot Zn^{2+} & & \end{array} \qquad \text{(III)}$$

$$\overset{K_{d,R \cdot Zn^{2+}}}{}$$

In scheme III, $K_{i,AR}$ is the dissociation constant for Zn^{2+} binding to the GABA-bound channel and $K_{d,R \cdot Zn^{2+}}$ is the dissociation constant for GABA binding to the Zn^{2+}-bound channel. For the sake of discussion, the channel opening (k_o) and closing (k_c) rates have been included in scheme III. Whether Zn^{2+} can also bind to open channels (AR^*) can be tested with a single-channel kinetic analysis. For an open channel blocker, a dependence of the mean open time on Zn^{2+}

concentration would be predicted. Such an analysis has been performed for Zn^{2+} antagonism of GABA-activated channels from cultured rat sympathetic, cerebellar, and hippocampal neurons (24, 33). These investigations demonstrated a decrease in the frequency of channel openings with Zn^{2+} and not a decrease in the mean open time, suggesting that Zn^{2+} is not an open channel blocker of GABA-activated channels but, rather, exerts its antagonism through a negative allosteric effect (24, 25, 33).

In scheme III, if the K_i and K_d values are not equivalent, the antagonism may appear mixed (34). For example, for $K_{i,R} \ll K_{i,AR}$ the antagonism would appear predominately competitive at lower Zn^{2+} concentrations. At higher Zn^{2+} concentrations, however, the transition rate from AR to AR·Zn^{2+} increases, approaching k_o, and the noncompetitive antagonism becomes prevalent.

Differences in the binding affinities (K_d and K_i values) and rate constants (k_c and k_o) in scheme III could affect the apparent mechanism of antagonism and may account, at least in part, for the reported variability in the effects of Zn^{2+} on different GABA-activated channel subtypes. For example, ρ1 GABA-activated channels activate and deactivate more slowly than heteromeric GABA-activated channels (9), which could affect the apparent mechanism of antagonism. In addition, ρ1 GABA-activated channels show very little desensitization, even at high GABA concentrations (9, 15). Thus, the current decay in the presence of Zn^{2+} could be attributed to Zn^{2+} antagonism alone, rather than a combination of Zn^{2+} antagonism and channel desensitization.

One key question that remains unanswered is whether the two putative Zn^{2+} binding steps, with different affinities, are at the same binding site. For example, there could be two separate Zn^{2+} binding sites, one that has high affinity and is competitive and another that has lower affinity and is non competitive. Alternatively, there may be only one Zn^{2+} binding site but its affinity differs depending on whether GABA is unbound (R) or bound (AR) to the receptor. A mutational analysis attempting to differentially affect the two components of antagonism may help to resolve this issue.

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