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Negative Modulation of the γ-Aminobutyric Acid Response by Extracellular Zinc

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SUMMARY

We have studied the effects of divalent cations on the γ-aminobutyric acid (GABA) response of voltage-clamped spinal cord neurons, using the whole-cell recording configuration. Zn, Cd, Ni, and Mn (but not Ba, Ca, or Mg) inhibit GABA-induced whole-cell currents when applied extracellularly. Although Zn is an effective inhibitor when applied extracellularly, it is ineffective when applied intracellularly. Inhibition by these cations is mediated by a common saturable recognition site that is distinct from the recognition sites for GABA, benzodiazepines, barbiturates, picrotoxin, or steroids. The maximal inhibition, or efficacy of inhibition, of GABA-induced currents is greater for Zn than for Cd, Ni, or Mn. The order of potency is Cd > Zn ≫ Ni ≫ Mn. Inhibition by Zn is partially surmountable by GABA, consistent with a decrease in both the maximum response and the affinity for GABA. The dose-response curve for inhibition of the GABA response by Zn is shifted to the right at a high GABA concentration but is unaffected by the presence of chlordiazepoxide, pentobarbital, or 5β-pregnan-3α-ol-20-one. The results are consistent with a model in which a Zn-sensitive modulatory site exerts negative allosteric control over GABA receptor function.

The circuitry of the vertebrate central nervous system is formed by interconnecting networks of neuronal pathways, and subtle alterations in synaptic transmission can have profound effects on the activity of a given pathway. In this way, neuromodulators can exert control over nervous system function. Since the discovery that benzodiazepines potentiate GABA sensitivity (1, 2), we have been exploring the pharmacological and cell biological mechanisms by which GABA receptor function is modulated (3, 4). In the present study, we focus on the modulation of the GABA response by extracellular zinc and other divalent cations.

Zinc is essential for the proper development of the nervous system (5). It is found in cerebrospinal fluid (6, 7) and throughout the brain, being most concentrated in the cortex and hippocampus (8). Of particular interest is the finding that Zn[1] is concentrated in synaptic terminals (9) and released, with electrical activity, in sufficient quantity (10) to play a potential role in neurotransmission (11). Moreover, Zn and certain other metal cations, such as Cd, Ni, and Co, inhibit the GABA response of neurons in a variety of organisms (12–16), and intraventricular injection of Zn causes seizures in rats (17, 18).

Separate modulator binding sites for benzodiazepines, barbiturates, and steroids are known to exist on the GABA receptor/chloride ionophore complex (19, 20). Heat-inactivation studies (21, 22) and radioligand-binding studies (23, 24) suggest the presence of a divalent cation binding site on the GABA receptor complex. The goal of this study is to determine whether a specific divalent cation recognition site mediates inhibition of the GABA response by Zn.

We find that Zn decreases both the potency of GABA and the maximum response to GABA, by acting upon a specific divalent cation modulatory site associated with the $GABA_A$ receptor. The site is clearly extracellular and is distinct from the benzodiazepine, barbiturate, picrotoxin, and steroid recognition sites. The results are discussed in terms of an allosteric model of receptor modulation.

Materials and Methods

Chemicals. All drugs used in electrophysiology experiments were obtained from Sigma, except chlordiazepoxide (gift of Hoffman-La Roche), SR-95531 (Research Biochemicals), and pregnenolone sulfate (Steraloids). Divalent cations (chloride salts) and other chemicals were obtained from Sigma or Fisher.

Spinal cord cultures. Spinal cord cultures were prepared from 7-day chick embryos, as previously described (25), and maintained in Eagle's minimum essential medium, supplemented to a final concentration of 2.4 mM glutamine, 24 mM KCl, 22 mM glucose, 10% heat-inactivated horse serum, 2.5% chick embryo extract, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cytosine arabinoside (0.1 μM) was used for 24 hr to control non-neuronal cell proliferation. Cultures were used in experiments 2–5 weeks after plating.

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[1] Valence notation is omitted for divalent cations, e.g., Zn represents Zn^{2+}.

ABBREVIATIONS: GABA, γ-aminobutyric acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid.

Electrophysiology. Cultures were washed three times with recording buffer (in mM: 150 NaCl, 4 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, pH 7.2 with NaOH) and studied using the whole-cell voltage-clamp configuration (26). Recordings were made at room temperature (20–22°) on the stage of an inverted phase-contrast microscope, using a Yale Mk V or an Axopatch 1B amplifier. Currents were filtered using an eight- or four-pole Bessel filter (1–5 kHz, −3 dB) and recorded on an MFE 1200 chart recorder or digitized (16–40 msec/point) using a microcomputer-based on-line data acquisition system (pClamp; Axon Instruments).

Patch electrodes had tip openings of about 2 μm and resistances of 3–8 MΩ when filled with standard electrode buffer (in mM: 140 KCl, 3 NaCl, 1 $MgCl_2$, 11 EGTA, 10 HEPES, pH 7.2 with KOH). Electrodes were prepared from 100-μl borosilicate micropipets, using a vertical David Kopf electrode puller. After partial compensation, series resistances were between 8 and 12 MΩ. When large GABA-induced currents were obtained, only those for which the calculated potential drop across the series resistance was <10 mV were used. For experiments in which high concentrations of GABA were used, the Cl^- concentration in the electrode was decreased from 150 mM to 20 mM by replacement with potassium gluconate. This reduced the driving force on Cl^- (i.e., the difference between the holding potential and the Cl^- reversal potential), thereby limiting the magnitude of the GABA-induced current, so that the potential drop across the series resistance remained within acceptable limits. Mg-ATP (4 mM) was also added to the electrode buffer, to reduce run-down seen with responses to high GABA concentrations (27). The membrane potential was held at −70 mV for low GABA concentrations (3–5 μM) and at −50 to −70 mV for higher GABA concentrations. Resting potentials of −50 to −70 mV and input resistances of 100–500 MΩ were typical. The study of a particular cell was terminated if the resting potential became less negative than −45 mV or the current required to hold the membrane potential at −70 mV exceeded 250 pA.

Drug application. Cations and drugs were dissolved in recording buffer and applied to individual neurons by pressure ejection (15 psi) from a seven-barrel pipette, with tip diameter of 3–5 μm, positioned no more than 50 μm from the cell body. Under these conditions, the drug solution ejected from the pressure pipette rapidly replaces the solution surrounding the target neuron, with <10% dilution (28). One barrel of the pressure pipette contained recording buffer, and application of GABA was normally preceded by a 20-sec pulse of buffer and followed by a 10-sec buffer pulse. For experiments involving very high concentrations of GABA (≥100 μM), the test neuron was continuously bathed in a flow of buffer from the pressure pipette, except when other solutions were being applied, to prevent desensitization due to leakage of GABA from the high concentration barrel. Divalent cations, 5β-pregnan-3α-ol-20-one, pentobarbital, and pregnenolone sulfate were applied simultaneously with GABA, mixed together in the same barrel of the pressure pipette. Chlordiazepoxide was applied as a 20-sec pulse immediately before application of GABA. 5β-Pregnan-3α-ol-20-one, pentobarbital, and pregnenolone sulfate were initially dissolved in dimethyl sulfoxide and diluted with recording buffer (final dimethyl sulfoxide concentration, ≤0.5%).

Desensitization increases with increasing GABA response (29). Therefore, the slow application of a high concentration of GABA can result in an underestimation of the peak response. For this reason, responses to GABA concentrations of 100 μM or more were excluded from study if >200 msec were required to reach 90% of the peak response. This criterion gives a reliable measure of the maximum GABA response, because the response to 1000 μM GABA was within 10% of the response to 100 μM GABA.

Data analysis. We calculated the percentage of inhibition or enhancement of the GABA response (α) due to a single modulator as follows:

$$\alpha = \left(\frac{I'}{I} - 1\right) \times 100$$

where I is the response to GABA alone and I' is the response to GABA plus a single modulator. To study modulator interactions, we also examined how the response to GABA in the presence of one modulator was affected by the addition of a second modulator. For example, the effect of Zn in the presence of Cd would be expressed as $\alpha_{Zn(Cd)} = (I''/I' - 1) \times 100$, where I' is the GABA response in the presence of Cd alone ($I_{GABA + Cd}$) and I'' is the response in the presence of both Cd and Zn ($I_{GABA + Cd + Zn}$).

To determine dose-response curves for modulators of the GABA response, pooled data for each modulator concentration were weighted according to the inverse standard error of replicate observations and were fit, by nonlinear regression, to the logistic equation (30)

$$\alpha = \alpha_{max} \times \left[\frac{[\text{modulator}]^{n_H}}{[\text{modulator}]^{n_H} + EC_{50}^{n_H}}\right]$$

where α_{max} is the maximum percentage of change in the GABA response and n_H is a slope factor related to the Hill coefficient.

The maximum GABA-induced current and the GABA EC_{50} were estimated by fitting the GABA dose-response data to the logistic equation (30), by nonlinear regression. Because the maximum GABA-induced current varied widely from neuron to neuron, data from different neurons were not pooled; instead, data from each neuron were fit to the logistic equation individually. Only cells for which data were adequate to define the maximum GABA response were included.

Results

Responses to GABA of embryonic chick spinal cord neurons in primary monolayer tissue culture were measured using the whole-cell voltage-clamp recording method. Under our experimental conditions of symmetrical [Cl^-] and at a holding potential of −70 mV, pressure ejection of GABA onto a neuron induced an inward membrane current (Fig. 1).

Inhibition of the $GABA_A$ response by Zn and Cd. The GABA-induced current was inhibited when Zn or Cd was applied simultaneously with GABA (Fig. 1). The site of action of Zn is evidently extracellular, because no inhibition of the GABA response was observed when Zn was applied intracellularly by inclusion in the electrode buffer (Fig. 2). The onset of inhibition was rapid, as evinced by the fact that inhibition of the GABA response did not require, and was not increased by, prior exposure of the neuron to Zn or Cd. Recovery from inhibition was also rapid; the response to a subsequent application of GABA alone was unaffected (Fig. 1). Moreover, inhibition was observed only when GABA was applied in combination with Zn or Cd; application of Zn or Cd immediately before application of GABA produced no inhibition. Inhibition of the GABA response was also observed in the presence of Ni and Mn but is not a general property of divalent cations, because Ba produced no inhibition (Fig. 1B).

Concentration dependence of inhibition by Zn, Cd, and Ni. Inhibition of the GABA response by Zn, Cd, and Ni was incomplete, even when these cations were applied at concentrations near their solubility limits in the recording solution; 15% of the GABA-induced current was resistant to 3 mM Zn, whereas 29% was resistant to 5 mM Cd and 20% was resistant to 10 mM Ni (Table 1). The Cd-resistant current was mediated by the $GABA_A$ receptor, because it was abolished by SR-95531 (Fig. 1A).

In an effort to determine whether the absence of complete inhibition was due to incomplete saturation of the relevant cation binding sites, pooled data were used to construct dose-response curves for inhibition of the GABA response by Cd, Ni, and Zn (Fig. 3), and maximal inhibition was estimated by nonlinear regression, using the logistic equation (see Materials

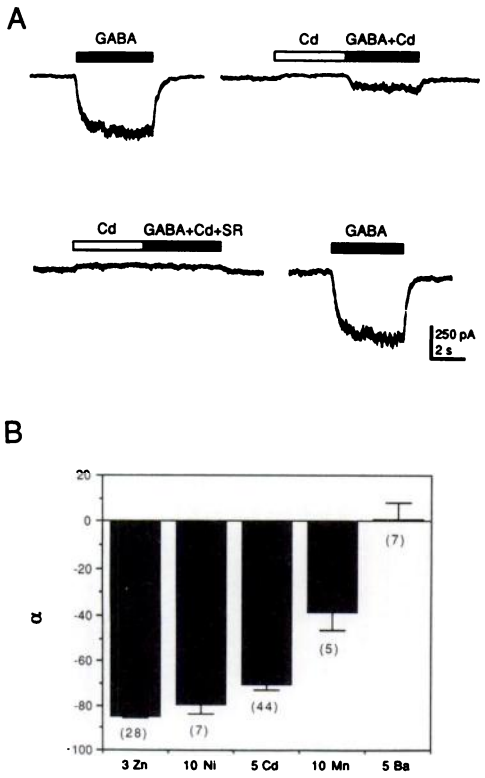

Fig. 1. Effects of divalent cations on the GABA response. A, Cd (5 nM) partially inhibits the response to 3 μM GABA. Cd alone has little effect on the whole-cell current, although a small outward current (≤10 pA) is occasionally observed, as in this example. The residual current is completely inhibited by 20 μM SR-95531 (*SR*), a competitive GABA antagonist. *Traces* were obtained from a single cell, with a 2–3-min wash between applications. Note complete recovery of the GABA response at the end of the recording session. *Horizontal bar above each trace*, period of drug application. Holding potential, −70 mV. B, Effects of various divalent cations on responses to GABA (3–5 μM). The concentration of each divalent cation (mM) is indicated on the *abscissa*. Percentage change (α) was determined as described in Materials and Methods. Number of cells is indicated in *parentheses*. *Error bars*, standard errors.

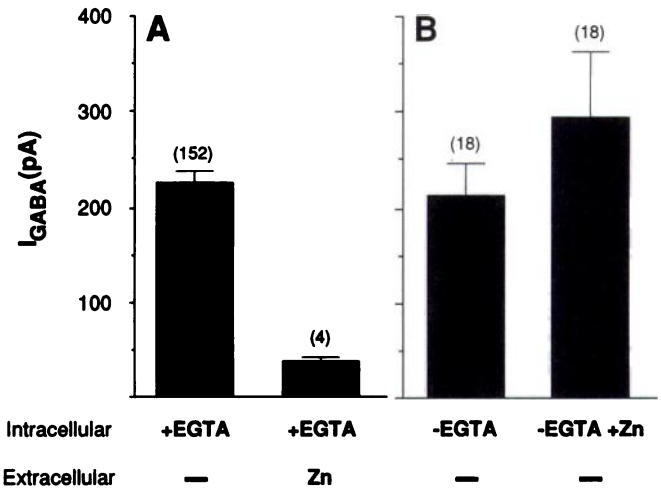

Fig. 2. Intracellular Zn does not inhibit the GABA response. A, Zn (1 mM) inhibits the response to 3 μM GABA. B, Addition of Zn (1 mM) to the intracellular (electrode) buffer does not inhibit the GABA response. EGTA in the electrode buffer was replaced with 11 mM potassium gluconate (−EGTA) to prevent chelation of Zn by EGTA. Removal of EGTA from the electrode buffer does not significantly affect the GABA response in the absence of Zn (compare *left bar* in A with *left bar* in B). Number of cells is indicated in *parentheses*. *Error bars*, standard errors.

TABLE 1

Divalent cation modulators of the GABA response display functional competition

Unless otherwise stated, concentrations are as follows: GABA, 3–5 μM; Zn, 3 mM; Ni, 10 mM; Cd, 5 mM; Mn, 10 mM; and Ba, 5 mM. Cd decreases the response to GABA alone (α_{Cd}) but increases the response to GABA in the presence of Zn ($\alpha_{Cd(Zn)}$). Fig. 4 shows typical traces from which α_{Cd} and $\alpha_{Cd(Zn)}$ were calculated. Zn substantially decreases the response to GABA alone (α_{Zn}) but has only a small inhibitory effect effect on the response to GABA plus Cd ($\alpha_{Zn(Cd)}$). Ni and Mn also decrease the response to GABA in the absence of Zn but increase the response in the presence of Zn. Ba alone has little effect on the response to GABA but increases the response in the presence of Zn or Cd. Values are means ± standard errors, and the number of cells is indicated in parentheses. Within each group, the first value is significantly different ($p < 0.001$) from subsequent values.

α_{Zn}	−85 ± 1 (28)*
$\alpha_{Zn(Cd)}$	−18 ± 10 (7)
α_{Ni}	−80 ± 4 (7)*
$\alpha_{Ni(Zn)}$[a]	+30 ± 8 (8)′
α_{Cd}	−71 ± 2 (44)*
$\alpha_{Cd(Zn)}$	+42 ± 20 (7)
$\alpha_{Cd(Zn)}$[a]	+78 ± 16 (14)*
$\alpha_{Cd(Ni)}$[b]	+3 ± 5 (6)
α_{Mn}	−39 ± 8 (5)′
$\alpha_{Mn(Zn)}$[a]	+94 ± 35 (6)ᵍ
α_{Ba}	+0.8 ± 6.8 (7)
$\alpha_{Ba(Zn)}$[c]	+32 ± 8 (19)*
$\alpha_{Ba(Cd)}$[d]	+89 ± 15 (25)*

[a] 20 μM GABA.
[b] 20 μM GABA, 3 mM Ni.
[c] 0.1 mM Zn.
[d] 0.5 mM Cd.
* Significantly different from 0 ($p < 0.001$).
′ Significantly different from 0 ($p < 0.01$).
ᵍ Significantly different from 0 ($p < 0.05$).

and Methods). This analysis predicts a maximal inhibition (α_{max}) of −88% by Zn, −84% by Ni, and −72% by Cd (Table 2), tending to support the view that complete inhibition of the GABA response by these ions is not attainable and suggesting that Zn has greater efficacy than Cd as an inhibitor of the GABA response. We did not attempt to fit the Mn dose-response curve, because Mn produced <40% inhibition at 10 mM, the highest concentration tested, but it is evident from inspection that the potency of Mn is substantially lower than that of Ni, whereas Ni is of lower potency than either Zn or Cd.

Divalent cation interactions. A more direct way to compare the action of Zn and Cd is to examine their effect when applied in combination. If incomplete inhibition of the GABA response reflects incomplete saturation of the relevant binding site, then the inhibition of the GABA response by Zn and Cd together should be greater than that produced by either Zn or Cd alone. If, instead, the lesser inhibition produced by Cd reflects a lower efficacy than that of Zn at a common site of action, then Zn and Cd should compete, and the inhibition produced by Zn and Cd in combination should be intermediate between that produced by Zn and Cd individually.

As shown in Fig. 4 and Table 1, the response to 20 μM GABA in the presence of both 3 mM Zn and 5 mM Cd was significantly greater than that in the presence of 3 mM Zn alone ($\alpha_{Cd(Zn)}$ = +78), indicating that Cd partially relieves the inhibition of the GABA response produced by Zn. Similarly, Ni (10 mM) or Mn (10 mM) partially relieved inhibition by Zn, whereas Cd was without effect in the presence of Ni. Ba (5 mM), which alone

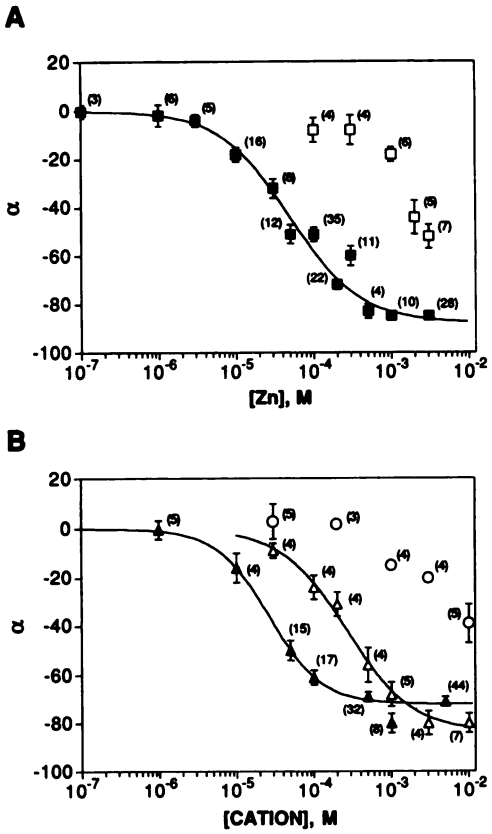

Fig. 3. Dose dependence of inhibition of the GABA response by Zn, Cd, Ni, and Mn. A, Effect of Zn on the response to 3–5 μM GABA (■) or 1000 μM GABA (□). Data are expressed as percentage change in the GABA response induced by Zn application. B, Effects of Cd (▲), Ni (△), and Mn (○) on the response to 3–5 μM GABA. *Curves*, results of nonlinear regression to the logistic equation (see Table 2 for parameters). Data are pooled from multiple experiments; the number of cells for each point is indicated in *parentheses*. *Error bars*, standard errors.

TABLE 2

Zn and Cd dose-response curves are unaltered by other $GABA_A$ receptor modulators

Dose-response curves were determined for inhibition of the GABA response by Zn and Cd in the presence and absence of 50 μM pentobarbital (PB), 1 μM 5β-pregnan-3α-ol-20-one (5β3α), or 300 μM chlordiazepoxide (CDPX). Zn dose-response curve was also determined in the presence of 100 μM Cd (see Fig. 5). Using pooled data from 4–10 neurons, the EC_{50}, maximal percentage of inhibition (α_{max}), and slope factor (n_H) for inhibition of the GABA response by Zn, Cd, and Ni were estimated by nonlinear regression, as described in Materials and Methods.

	EC_{50}	α_{max}	n_H
	μM		
α_{Zn}	50	−88	0.96
$\alpha_{Zn(PB)}$	86	−86	0.81
$\alpha_{Zn(5\beta3\alpha)}$[a]	80	−90	0.65
$\alpha_{Zn(Cd)}$	219	−85	0.96
α_{Cd}	27	−72	1.3
$\alpha_{Cd(CDPX)}$	26	−68	1.2
α_{Ni}	270	−84	1.0

[a] 1.5 μM GABA.

had no effect on the GABA response (Fig. 1B; Table 1), partially relieved inhibition of the GABA response by either Zn (0.1 mM) or Cd (0.5 mM).

To further explore the interaction between Zn and Cd, the dose-response curve for inhibition of the GABA response by Zn was studied in the presence of 100 μM Cd, which by itself produces about 60% inhibition of the GABA response. As shown in Fig. 5 and Table 2, the apparent EC_{50} for inhibition of the GABA response by Zn was increased by >4-fold in the presence of Cd, consistent with a competitive interaction between Zn and Cd.

The observation that Ba could reduce inhibition of the GABA response by Cd suggested the possibility that inhibition of the GABA response by Zn or Cd might be limited by other divalent cations, such as Ca and Mg, that are present in our standard recording buffer; however, this does not seem to be the case. Removal of Ca and Mg did not increase inhibition by Zn or Cd. Inhibition of the response to 3 μM GABA, in Ca- and Mg-free buffer, by 3 mM Zn [$\alpha_{Zn} = -81 \pm 3$ ($n = 5$)] or 5 mM Cd [$\alpha_{Cd} = -71 \pm 6$ ($n = 6$)] was similar to that in standard recording buffer (compare with Table 1).

Effect of concentration of GABA. Because the large current induced by a high concentration of GABA can create an unacceptably large potential drop across the recording electrode, the Cl^- concentration in the electrode was reduced from 150 mM to 20 mM (see Materials and Methods), to study the effects of Zn and Cd at elevated concentrations of GABA. This did not significantly alter the percentage of inhibition of the response to 10 μM GABA produced by 3 mM Zn [$\alpha_{Zn} = -86 \pm 3$ ($n = 6$) with 20 mM Cl^- and $\alpha_{Zn} = -80 \pm 3$ ($n = 6$) with 150 mM Cl^-].

As shown in Table 3, inhibition by Zn or Cd could be

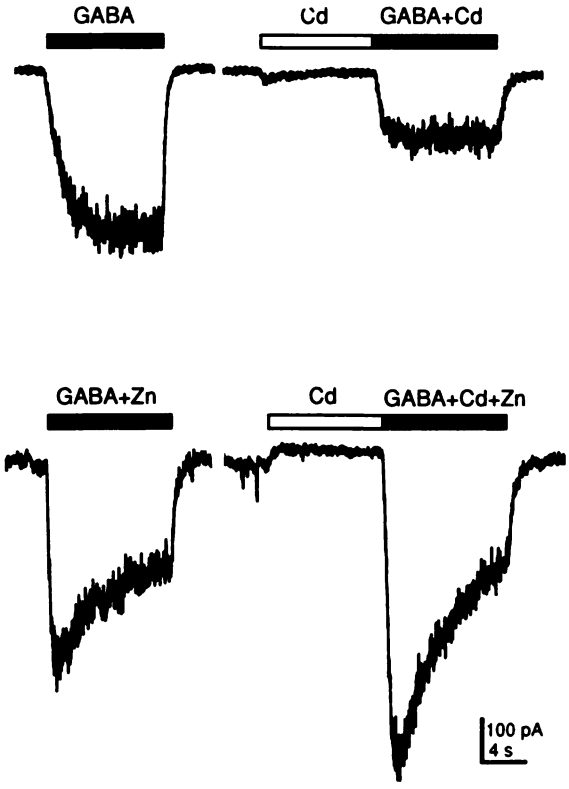

Fig. 4. Cd partially relieves inhibition of the GABA response by Zn. Cd (5 mM) reduces the response to 5 μM GABA (*upper traces*). Cd (5 mM) increases the response to 20 μM GABA plus 3 mM Zn (*lower traces*). *Traces* are from a single cell, with a 2–3-min wash between drug applications (see Table 1 for pooled data). Holding potential, −70 mV. The rapid desensitization in the lower traces is typical of the majority (9 of 17) of the neurons exposed to 20 μM GABA plus 3 mM Zn. Others showed little (3 of 17 neurons) or no (5 of 17 neurons) desensitization over the period of GABA application.

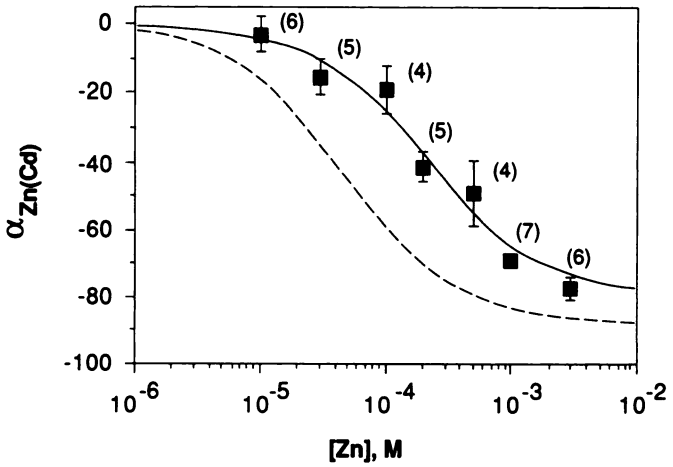

Fig. 5. Cd decreases the potency of Zn. Effect of Zn on the response to 5 μM GABA in the presence of 100 μM Cd. *Smooth curve through data points*, results of nonlinear regression to the logistic equation (see Table 2 for parameters). *Dashed curve*, fitted Zn dose-response curve in the absence of Cd, reproduced from Fig. 3A. *Points*, means of pooled data from multiple experiments, expressed as $\alpha_{Zn(Cd)}$, the percentage Zn-induced inhibition of the response to GABA plus 100 μM Cd. The number of cells for each point is indicated in *parentheses*. *Error bars*, standard errors.

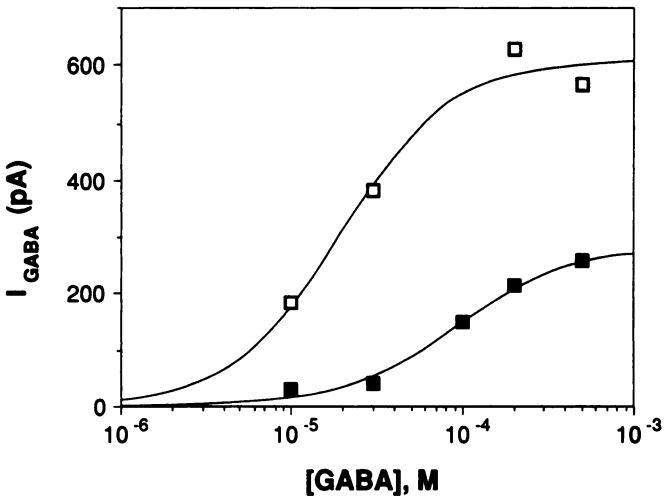

Fig. 6. Zn reduces the potency and efficacy of GABA. Currents induced by various GABA concentrations, from a single neuron, in the absence (□) and presence (■) of 3 mM Zn. Holding potential, −50 mV; intracellular [Cl^-], 20 mM; 4 mM Mg-ATP is included. *Curves*, results of nonlinear regression to the logistic equation. In addition to decreasing the maximum GABA response (see Table 3 for pooled data), Zn also decreases the apparent potency of GABA [EC_{50} for GABA, 36 ± 9 μM (n = 7) without Zn; 115 ± 19 μM (n = 7) with Zn].

TABLE 3

Zn- and Cd-induced inhibition is partially surmountable by GABA

Inhibition of the GABA response by 3 mM Zn or 5 mM Cd was determined at a series of GABA concentrations (μM). Values represent percentage of change in the GABA response (α) and are expressed as means ± standard errors. The number of cells studied at each concentration is indicated in parentheses. For both Zn and Cd, the difference in α at the highest and lowest concentrations of GABA is statistically significant ($p \leq 0.003$).

	3 GABA	5 GABA	20 GABA	500 GABA	1000 GABA
α_{Zn}	−84 ± 1 (19)	−87 ± 2 (9)	−77 ± 4 (8)	−57 ± 4 (10)	−52 ± 5 (7)
α_{Cd}	−71 ± 2 (30)	−70 ± 4 (11)	−55 ± 3 (7)	−55 ± 8 (6)	

TABLE 4

Zn and Cd do not interfere with the action of other GABA receptor modulators

The effects of picrotoxin (Pic) (100 μM), pregnenolone sulfate (PS) (50 μM), chlordiazepoxide (CDPX) (300 μM), pentobarbital (PB) (50 μM), or 5β-pregnan-3α-ol-20-one (5β3α) (1 μM) on the GABA response were determined in the presence and absence of 5 mM Cd or 3 mM Zn. Unless otherwise stated, the concentration of GABA was 3–5 μM. In all cases, the percentage of enhancement or inhibition of the GABA response was not significantly affected by the presence of Zn or Cd ($p > 0.05$, unpaired t test). Values are means ± standard errors, and the number of cells is indicated in parentheses.

α_{Pic}	−80 ± 5 (6)
$\alpha_{Pic(Cd)}$	−67 ± 8 (6)
α_{PS}	−85 ± 4 (7)
$\alpha_{PS(Zn)}$[a]	−78 ± 5 (7)
α_{CDPX}	+214 ± 34 (28)
$\alpha_{CDPX(Cd)}$	+293 ± 46 (9)
α_{PB}	+466 ± 86 (11)
$\alpha_{PB(Zn)}$	+410 ± 90 (8)
$\alpha_{5\beta3\alpha}$	+848 ± 380 (7)
$\alpha_{5\beta3\alpha(Cd)}$	+1060 ± 344 (7)

[a] 20 μM GABA.

partially, but not completely, surmounted by increasing the concentration of GABA from 3 μM to 1 mM. Consistent with this observation, examination of GABA dose-response curves reveals a significant increase in the GABA EC_{50} [from 36 ± 9 μM to 115 ± 19 μM (n = 7); $p < 0.005$] in the presence of 3 mM Zn, as well as a decrease in the maximum GABA-induced current (Fig. 6). The observation that Zn displaces the GABA dose-response curve to the right implies that increasing the concentration of GABA should lead to a reciprocal rightward shift of the Zn dose-response curve for inhibition of the GABA response. As expected, the inhibitory potency of Zn was reduced when the concentration of GABA was increased (Fig. 3A).

Effects of other modulators of the GABA response. The effects of positive and negative modulators of the GABA response were studied in the absence and presence of 3 mM Zn or 5 mM Cd. Although the response to GABA was greatly reduced in the presence of these high concentrations of Zn and Cd, the percentage of enhancement of the GABA response produced by chlordiazepoxide (300 μM), pentobarbital (50 μM), or 5β-pregnan-3α-ol-20-one (1 μM) was not significantly altered in the presence of Zn or Cd. Similarly, and in contrast to the results obtained with combinations of Zn and Cd, Ba, or Ni, the percentage of inhibition of the residual GABA response produced by picrotoxin (100 μM) or pregnenolone sulfate (50 μM) did not differ significantly from that observed in the absence of Zn and Cd (Table 4).

To test whether known positive modulators of the GABA response compete with Zn or Cd, Zn or Cd dose-response curves were obtained in the presence of chlordiazepoxide, pentobarbital, or 5β-pregnan-3α-ol-20-one. Neither the potency nor the efficacy of Zn or Cd as an inhibitor of the GABA response was substantially altered (Table 2) in the presence of these modulators.

Discussion

The response of the neuronal $GABA_A$ receptor to exogenously applied GABA is allosterically modulated by a wide variety of

substances that act at specific modulatory sites on the $GABA_A$ receptor (19, 20). Our results argue that inhibition of the GABA response by certain divalent metal cations, such as Zn and Cd, is mediated by a novel modulatory site that is distinct from previously identified modulatory sites associated with the $GABA_A$ receptor.

Inhibition of the $GABA_A$ receptor by metal cations such as Zn, Cd, Mn, and Ni could conceivably occur by four mechanisms, 1) formation of inactive complexes with GABA, 2) obstruction of the GABA binding site (competitive inhibition), 3) obstruction of the $GABA_A$ receptor-associated Cl^- ionophore (channel blockade), or 4) allosteric modulation at a distinct site.

Divalent cations can form complexes with amino acids, thereby reducing the concentration of free amino acid; however, inhibition by this mechanism is expected to be fully surmountable (12), whereas inhibition of the GABA response by Zn and Cd was not. Moreover, this hypothesis cannot account for the observation that mixtures of Zn with Cd, Ni, Mn, or Ba produced less inhibition than Zn alone.

Competition of Zn and Cd for the GABA binding site cannot account for several aspects of the observed results. In particular, competitive inhibition at a single class of binding sites cannot explain the failure to achieve complete inhibition of the GABA response, which was particularly apparent for Cd. Although the lack of complete inhibition could conceivably be due to a subpopulation of receptors to which these cations do not bind, this hypothesis also does not account for the inability to fully surmount inhibition by Zn or Cd by increasing the concentration of GABA. Finally, the competitive hypothesis cannot account for the partial relief of Zn-induced inhibition by Cd, Ni, Mn, or Ba.

Obstruction of an anion channel, such as the Cl^- ionophore, by cations such as Zn and Cd seems unlikely on theoretical grounds. In particular, the $GABA_A$ receptor is thought to bear positive charges close to the mouth of the channel that would deter entry of cations into the channel (31). The channel blockade hypothesis also cannot account for the observation that Ba fails to inhibit the GABA response yet reduces inhibition by Zn and Cd. Finally, the GABA concentration dependence of inhibition by Zn is the reverse of that which would be expected of a channel blocker; the potency of Zn decreased as the concentration of GABA was increased, whereas the potency of a channel blocker would be expected to increase due to the greater number of open channels.

Inhibition of the GABA response by Zn and Cd is most consistent with an allosteric mechanism of action, mediated by a specific modulatory site associated with the $GABA_A$ receptor. The difference in the maximum inhibition by Zn and Cd is consistent with a model in which these ions act at a common site but possess different intrinsic efficacies as allosteric inhibitors, such that Zn has greater efficacy than Cd. According to this hypothesis, Cd should reduce the inhibition produced by a high concentration of Zn, in the same way that a partial agonist reduces the response to a full agonist by displacing it from its binding site. As predicted, inhibition of the GABA response by Zn was partially relieved by Cd.

As a further test of the competitive hypothesis for the Zn-Cd interaction, the dose-response curve for inhibition of the GABA response by Zn was determined in the presence of 100 μM Cd, a concentration sufficient to inhibit the GABA response by about 60%. Taking the experimentally determined EC_{50} of 27 μM for Cd to be its effective K_d, the apparent EC_{50} for Zn should be increased by a factor of $1 + 100\ \mu M/27\ \mu M = 4.7$, yielding a predicted Zn EC_{50} of 235 μM. This agrees well with the experimentally determined EC_{50} of 219 μM for Zn in the presence of 100 μM Cd.

Ni is less potent than either Cd or Zn as an inhibitor of the GABA response, and Mn is less potent than Ni. Inhibition of the GABA response by Zn was partially relieved by Ni, Mn, and Ba, arguing that these cations also have lower efficacy than Zn as inhibitors of the GABA response. The fact that Ba did not directly inhibit the GABA response but relieved inhibition by Zn and Cd suggests that Ba binds to the divalent cation modulatory site but lacks efficacy as an inhibitor of the GABA response.

Recombinant $GABA_A$ receptors expressed from only α and β subunits are sensitive to inhibition by Zn or Cd but do not exhibit modulation of the GABA response by benzodiazepines. If messenger for the γ subunit is included in addition to α and β, sensitivity to benzodiazepines appears (32), but inhibition by Zn is lost (33). In contrast, the GABA response of chick spinal cord neurons is both positively modulated by benzodiazepines, such as chlordiazepoxide, and negatively modulated by Zn and Cd. Do chick spinal cord neurons possess a mixture of $GABA_A$ receptors, some sensitive to Zn and Cd but not benzodiazepines and others sensitive to benzodiazepines but not Zn and Cd? The present results exclude this explanation. If Cd-sensitive $GABA_A$ receptors constitute a distinct subpopulation of benzodiazepine-insensitive receptors, then the percentage of enhancement of the GABA response by chlordiazepoxide should be increased when the masking effect of this benzodiazepine-insensitive subpopulation is reduced by Cd. Contrary to this expectation, the percentage of enhancement of the GABA response produced by chlordiazepoxide was unaltered by the inclusion of 5 mM Cd, a concentration that greatly reduces the total GABA-induced current.

Although chlordiazepoxide, pentobarbital, and 5β-pregnan-3α-ol-20-one increase the potency of GABA, they did not significantly affect the potency of Cd or Zn. This suggests that the mechanism by which these compounds enhance the GABA response is independent of that by which Zn and Cd inhibit the GABA response.

Inhibition of the response to GABA by various divalent cations has been reported in lobster (12), turtle (15), frog (14), fetal mouse (13), fetal rat (34), and embryonic kidney cells expressing recombinant rat $GABA_A$ receptors (33). Zn, Cd, Ni, Mn, Co, and Cu have all been found to inhibit the response to GABA in at least one species, whereas Ca, Mg, and Ba were consistently without effect when applied extracellularly (12, 14, 15). Increased intracellular Ca reduces the affinity of the $GABA_A$ receptor (35), but this is not likely to be the same mechanism by which Zn acts, because intracellular Zn did not inhibit the GABA response. Inhibition of the GABA response by divalent cations has consistently been found to be reversible, with little or no voltage dependence (14, 15, 33, 34).

Although inhibition of the GABA response by divalent cations has been widely reported, it is clear that divalent cations do not affect all $GABA_A$ receptors identically. Studies of the mechanism of divalent cation-induced inhibition of $GABA_A$ receptor function have yielded conflicting results. Some studies report pure noncompetitive inhibition (15, 33, 34), whereas in

frog sensory neurons Zn decreased the potency of GABA without affecting the maximum response. $GABA_A$ receptors of rat CA3 hippocampal neurons were not inhibited by Zn, although there is evidence that endogenous Zn plays a role in hippocampal neurotransmission by inhibiting $GABA_B$ receptors (11). The inhibitory effect of Zn decreased with age in the rat superior cervical ganglion (34) and was not observed in adult rat cortex (36, 37). These differences most likely reflect $GABA_A$ receptor heterogeneity.

Zn-protein interactions range from very stable metalloproteins, with stability constants of 10^9 to 10^{11} M^{-1} (38–41), to less stable complexes formed by such proteins as albumin or insulin, with stability constants of 10^5 to 10^7 M^{-1} (42, 43). Because inhibition of the GABA response occurs at micromolar concentrations, the GABA receptor-Zn interaction more closely resembles the latter group.

$GABA_A$ receptor sequences reveal numerous residues that could contribute to a Zn binding site (32, 44). Zn-protein complexes commonly involve coordination sites employing histidine, cysteine, aspartate, or glutamate (45). Both α and γ subunit sequences of the $GABA_A$ receptor include a conserved combination of residues (Cys-138, His-141, His-151, and Cys-153; α1 numbering) that has been suggested to resemble a "Zn finger" (16). However, the close proximity of the last two residues is not typical of Zn fingers (46, 47), and the cysteines are believed to form a disulfide bond analogous to that of the nicotinic acetylcholine receptor (31), in which case they would be unavailable to contribute to Zn binding.

Although most protein coordination sites for Zn involve amino acids from a single polypeptide chain (45), several exceptions exist in which coordination sites involve amino acids from different polypeptides (48–50). A Zn modulatory site on the $GABA_A$ receptor involving residues from adjacent subunits would likely be highly sensitive to differences in subunit composition. Such a model could explain the diversity of Zn effects on receptors derived from different species, as well as the finding that recombinant receptors including a γ subunit are insensitive to Zn (33). Moreover, a coordination site involving adjacent subunits could allow for independence of the mechanisms by which Zn and other modulators function. It is interesting to speculate that Zn might exert its modulatory effect by causing changes in quaternary structure, i.e., subunit interactions, whereas other modulators, such as benzodiazepines, barbiturates, and steroids, might primarily affect the tertiary structure of individual subunits.

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