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Zinc Selectively Inhibits Flux through Benzodiazepine-Insensitive γ-Aminobutyric Acid Chloride Channels in Cortical and Cerebellar Microsacs

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SUMMARY

The effects of Zn^{2+} on the activity of γ-aminobutyric acid $(GABA)_A$ receptor-Cl^- ionophore complexes found in adult rat cortex and cerebellum were tested by measuring $^{36}Cl^-$ influx into microsacs. In both preparations, the concentration-response curves were biphasic, with 25% of the cerebellar and 20% of the cortical Cl^- flux being blocked by less than 10 μM Zn^{2+} and 45% of the cerebellar and 50% of the cortical flux being blocked by concentrations of Zn^{2+} exceeding 10 μM. Zn^{2+} (100 μM) did not affect basal Cl^- flux but inhibited that stimulated by 100 μM GABA in a noncompetitive manner. The ability of 1 μM flunitrazepam to enhance Cl^- flux was unaffected by 100 μM Zn^{2+}. These results demonstrate that, in adult rat cerebellum and cortex, there are three populations of $GABA_A$ receptors, two that are sensitive to Zn^{2+} and insensitive to benzodiazepines (BDZ) and the remainder that are the reverse, i.e., insensitive to Zn^{2+} but fully sensitive to BDZ enhancement. This result is consistent with the idea that Zn^{2+} blocks only those $GABA_A$ receptor-Cl^- ionophore complexes that lack a γ subunit, which is required for modulation by BDZ. The results obtained in this study also show that the proportion of Zn^{2+}-sensitive GABA receptors is substantial, suggesting that they play an important role in the functioning of the adult central nervous system.

Micromolar concentrations of the transitional metal zinc have been shown to modulate the flow of chloride through $GABA_A$ chloride channels in a variety of neuronal systems, reducing it in some, such as cultured hippocampal neurons (1, 2), frog dorsal root ganglion cells (3), and cat spinal cord afferent axon terminals (4), enhancing it in others, such as adult guinea pig cortical neurons (5) and CA1 and CA3 hippocampal neurons (6), and causing bursting of putative GABAergic interneurons in rat neocortex (7).

Each $GABA_A$ receptor-ionophore complex is thought to be made up of five subunits. These subunits can be divided into α, β, γ, and δ subfamilies on the basis of their homology; however, the stoichiometry of subunits in the natural receptors is unknown (8). Reconstitution of GABA receptors in *Xenopus* oocytes or transfected fibroblasts has demonstrated that their functional properties are determined by the subunit composition, although only a fraction of the modulating factors are presently known. The species of α subunit primarily determines the BDZ pharmacology (9), but high affinity BDZ binding requires the presence of a γ subunit (10), with the γ2 subunit usually being the best of the γ subunits at bestowing BDZ efficacy, as determined by measurement of Cl^- currents (11).

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There have been recent reports that, when α1 or α3 and β1 or β2 subunits are singly expressed or coexpressed, the conductance through the resulting receptor-channel complex is completely inhibited by micromolar concentrations of Zn^{2+} (12). However, when γ1 or γ2 subunits are also incorporated the complex is virtually insensitive to the blocking action of Zn^{2+} (12, 13). This information, coupled with the knowledge that a γ subunit is required for BDZ modulation of GABA-mediated chloride conductance, would suggest that BDZ-sensitive GABA receptors should be insensitive to Zn^{2+} (6, 13, 14), whereas BDZ-insensitive GABA receptors should be blocked by Zn^{2+}. If such distinct populations of $GABA_A/Cl^-$ channel receptors could be further verified in specific brain regions, an enhanced effect of BDZ modulation of BDZ-sensitive $GABA_A/Cl^-$ channels could be observed by using Zn^{2+} to block the GABA-mediated Cl^- ion flux associated with BDZ-insensitive receptors.

There seems to be a change in the Zn^{2+} sensitivity of GABA receptors during development. As superior cervical ganglion cells mature, the GABA receptor population has been shown to become less sensitive to blocking by Zn^{2+} (6, 13), indicating a developmental change in the functional properties of $GABA_A$ receptors. Thus, although a number of studies have definitively demonstrated that the GABA responses of embryonic neurons

ABBREVIATIONS: GABA, γ-aminobutyric acid; BDZ, benzodiazepine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ANOVA, analysis of variance.

of sympathetic ganglia (13) and hippocampus (1, 2) are more sensitive to Zn^{2+} (13, 6), there are relatively few reports that Zn^{2+}-sensitive $GABA_A$ receptors exist in the adult central nervous system (4). There is, however, evidence from binding studies that BDZ-insensitive $GABA_A$ receptors exist; the density of GABA binding sites is greater than that of BDZ binding sites (15). *In situ* hybridization studies (16, 17) have also identified areas of the brain that have little or no mRNA for γ subunits. Therefore, it is possible that $GABA_A$ receptors made by the neurons of that area are not only BDZ insensitive but also Zn^{2+} sensitive.

The objectives of this study were to determine whether, indeed, Zn^{2+}-sensitive GABA receptors exist in the adult rat cortex and cerebellum. Conventional binding studies using [^{3}H] muscimol would not likely yield useful information, because Zn^{2+} blocks, in a noncompetitive manner, the ability of GABA to open channels (18, 19). Instead, the ability of Zn^{2+} to affect GABA/Cl^- channel function was assessed using the technique of $^{36}Cl^-$ influx into microsacs. This technique should be able to examine all $GABA_A$/Cl^- channels, even those that are on very small neurons, such as interneurons, or on distal dendrites of larger neurons that are difficult to examine using electrophysiological techniques. The cortex was chosen for study because BDZs have been shown to reliably modulate GABA-evoked Cl^- flux in this tissue (20). The cerebellum was chosen for study because this region expresses a limited number of GABA receptor subtype variants (16, 17) and has predominantly type I BDZ receptor pharmacology for BDZ agonists (21, 22).

Materials and Methods

Chloride Flux

Preparation of microsacs. A modified version of the method of Ngur *et al.* (23) was followed. Anesthetized Sprague-Dawley rats were decapitated, the brains were removed, and the cortices and cerebellum were dissected, separately homogenized in glass/Teflon homogenizers, and then centrifuged for 5 min at 15,000 × *g*. The buffer contained the following (in mM): NaCl, 145; KCl, 5; $CaCl_2$, 1; glucose, 10; HEPES, 10; and $MgSO_4$, 1. The pH was adjusted to 7.5 with Tris base. The pellet was washed twice, by resuspension (with vortexing) followed by centrifugation. The final tissue concentration was adjusted to 15 mg of wet weight/assay tube.

Chloride flux assay. The tissue was preincubated at 30° for 15 min before the addition of 200 μl of prewarmed (30°) GABA, Zn^{2+}, and $^{36}Cl^-$ (0.5 μCi/tube) to initiate $^{36}Cl^-$ flux. When required, flunitrazepam was added at the beginning of the preincubation period. To reduce possible solvent effects, the BDZ was first dissolved in 1.2 M HCl to make a 10 mM stock and then diluted 1000-fold in buffer that had a compensating alkalinity. At the highest BDZ concentration tested, 1 μM, this method increased the concentration of Cl^- by 0.12 mM in the final incubation buffer. After a 5- or 3-sec incubation, the Cl^- flux was terminated by the addition of 4 ml of ice-cold stopping buffer containing bicuculline or picrotoxin (100 μM) and furosemide (100 μM) and filtration on Whatman GF/C filters that had been presoaked in 0.1% polyethyleneimine in 0.9% saline solution, using a Hoeffer manifold. Filters were washed twice with stopping buffer and the radioactivity was counted. The amount of protein in microsacs was determined by the method of Lowry *et al.* (24).

The maximal effect, EC_{50}, and slope values were computed by fitting the data to the equation:

$$Y = \frac{(A - D) + D}{1 + (X/C)^B} \quad (1)$$

where A represents the expected maximal response, B is the slope factor, C is the EC_{50}, and D is the minimal response.

Receptor Binding Studies

Preparation of membranes. Rats were sacrificed by decapitation, and their brains were removed and dissected. The cerebellum was homogenized with a Polytron homogenizer in 40 volumes of 50 mM Tris·HCl, pH 7.7 at 0°, and were centrifuged at 20,000 × *g* for 10 min. The pellets were rehomogenized and centrifuged twice, frozen, thawed, washed an additional two times, and stored at −80°. For binding assays, membranes were suspended in assay buffer (50 mM Tris·HCl, pH 7.7 at 0°) to a tissue concentration of 4.5 mg of wet weight/ml.

Receptor binding assay. Membranes or microsacs (1 ml) were incubated in triplicate, with or without 100 μM $ZnCl_2$, with 0.5 nM [^{3}H] Ro15–1788 and eight concentrations of Ro15–1788 or flunitrazepam, in a total volume of 2 ml, for 90 min. Nonspecific binding was determined in the presence of 1 μM Ro15–1788. The reaction was terminated by filtration (Brandel cell harvester) through glass fiber filters (Whatman GF/B), followed by three 5-ml washes with ice-cold buffer. Radioactivity retained on the filters was determined by liquid scintillation counting.

Drugs

Flunitrazepam and Ro15–1788 were generously provided by Hoffmann-LaRoche. [^{3}H]Ro15–1788 and $^{36}Cl^-$ were purchased from DuPont-NEN.

Analysis

For statistical analysis of Cl^- flux studies, ANOVA followed by the Fisher exact test, or Student *t* test where appropriate, was performed using Statview.

Results

Effect of Zn^{2+} on maximal GABA-stimulated Cl^- flux. Fig. 1 shows the concentration-response curves for Zn^{2+} effects on the Cl^- flux evoked by 100 μM GABA, for both cortical and cerebellar microsacs. Both curves were clearly biphasic. One component, representing about 20% of the flux into cortical microsacs and 25% of the flux into cerebellar microsacs, was maximally blocked by 1–10 μM Zn^{2+}. The other components, representing at least 45% of the cerebellar total flux and 50% of the cortical total flux, were insensitive to Zn^{2+} concentrations lower than 10 μM. The basal flux rate in both tissues measured in the absence of added GABA was unaffected (ratio of basal

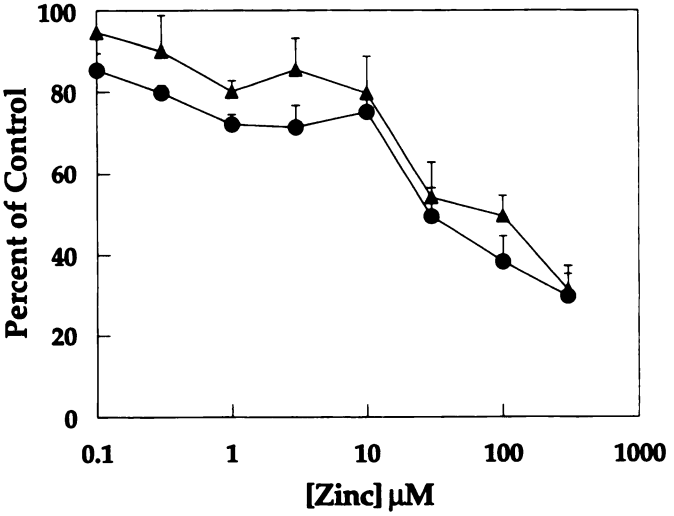

Fig. 1. Zn^{2+} concentration-response curve for the Cl^- flux evoked by 100 μM GABA in cortical (●) and cerebellar (▲) microsacs. The *points*, expressed as percentage of the Zn^{2+} value, represent an average of four to 17 experiments.

flux with and without 100 μM Zn^{2+}: cortex, 114 ± 13%, 18 experiments; cerebellum, 97 ± 9%, 25 experiments).

Effect of Zn^{2+} on GABA concentration-response curve for cerebellar microsacs. In an effort to determine the type of inhibition caused by Zn^{2+}, the effect of Zn^{2+} on the GABA concentration-response curve was investigated. Fig. 2A shows the effect of 100 μM Zn^{2+} on GABA-induced Cl^- flux into cerebellar microsacs evoked by varying concentrations of GABA. Table 1 contains the values for the maximal Cl^- flux, EC_{50}, and slope obtained by using eq. 1 to fit the results shown in Fig. 2A. A concentration of 100 μM Zn^{2+} was chosen because it maximally inhibited GABA-activated membrane currents in kidney cells transfected with $\alpha1\beta1$ subunits (13). Independently of the presence of Zn^{2+}, the GABA concentration-response curve reached a maximum at 100 μM GABA, but the maximum effect was greatly diminished in the presence of Zn^{2+}. From the data presented in Table 1 for the characteristics of the GABA concentration-response curves with and without 100 μM Zn^{2+}, it is evident that Zn^{2+} reduced the maximal response and increased the EC_{50} of the GABA concentration-response curve, whereas the slope was not significantly changed. The suppression of maximal effect is indicative of a noncompetitive mechanism of inhibition by Zn^{2+}. This noncompetitive effect is confirmed by the lack of GABA concentration dependence of the ratio of Cl^- flux with and without Zn^{2+} (Fig. 2B). As shown in Fig. 2B, the proportion of Cl^- flux blocked by Zn^{2+} remained constant over the entire concentration range of GABA.

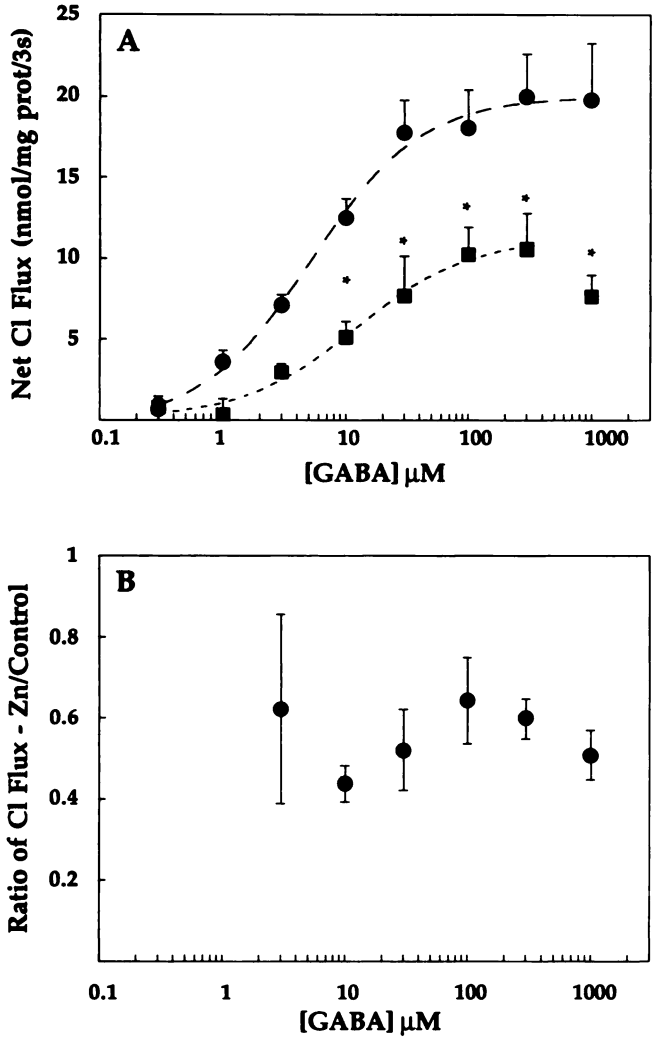

Fig. 2. A, GABA concentration-response curve in cerebellar microsacs in the absence (●) and presence (■) of 100 μM Zn^{2+}. The *points* represent the mean ± standard error of four to 15 experiments. Flux is expressed as increase over Cl^- flux in the nominal absence of GABA. The *curves* were computed by fitting the data to eq. 1. B, Plot of the ratio of Cl^- flux in the presence and absence of Zn^{2+} as a function of GABA concentration, calculated from individual experiments shown in A. ANOVA analysis found no difference between any of the ratios.

TABLE 1

Characteristics of GABA concentration-response curves for cerebellar microsacs in the absence and presence of 100 μM Zn^{2+}

Values were calculated using eq. 1 as described in Materials and Methods.

Condition	Maximal effect	EC_{50}	Slope
	nmol/mg of protein/3 sec	*μM*	
Control	20.0 ± 0.6	5.24 ± 1.0	0.97 ± 0.17
100 μM Zn^{2+}[a]	11.3 ± 1.0	12.2 ± 3.5	0.94 ± 0.3

[a] The value for 1000 μM GABA in the presence of Zn^{2+} was not included in the curve fitting.

Effect of Zn^{2+} on flunitrazepam concentration-response curve. Fig. 3A shows the flunitrazepam concentration-response curve for the effect on enhancement of Cl^- flux mediated by 10 μM GABA in the presence and absence of Zn^{2+}. Although flunitrazepam enhanced the GABA-stimulated flux in both instances, the relative enhancement over vehicle was greatly increased in the presence of Zn^{2+}. When the absolute differences in Cl^- flux with and without Zn^{2+} at each concentration of flunitrazepam were plotted, (Fig. 3B), it was evident that Zn^{2+} produced a constant decrease throughout the flunitrazepam concentration range.

Effect of Zn^{2+} on the absolute amplitude of the flunitrazepam enhancement of GABA-stimulated Cl^- flux. To ascertain whether Zn^{2+} affected the functioning of BDZ-sensitive $GABA_A$ receptor/Cl^- channels, the ability of 1 μM flunitrazepam to enhance the action of 10 and 100 μM GABA was examined in the presence and absence of 100 μM Zn^{2+}, in the cerebellum (Fig. 4) and the cortex (Fig. 5). As seen in Figs. 4 and 5, in both tissues the absolute amounts of BDZ enhancement with and without Zn^{2+} were similar. Thus, the presence of Zn^{2+} does not affect the ability of flunitrazepam to enhance Cl^- flux stimulated by 10 μM GABA. As shown in Table 2, in both tissues Zn^{2+} did not alter the absolute enhancement produced by 1 μM flunitrazepam on Cl^- ion flux stimulated by 10 μM GABA in cerebellar and cortical microsacs. At 100 μM GABA, a concentration that fully activated Cl^- flux in cerebellum (Fig. 2), flunitrazepam caused no enhancement in either the absence and presence of Zn^{2+}. However, in cortical microsacs, flunitrazepam significantly increased Cl^- flux evoked by the high concentration of GABA in the presence of Zn^{2+}, an effect that was absent under control conditions. These results provide evidence that the BDZ-sensitive $GABA_A$ receptors were minimally affected by Zn^{2+}.

Although the absolute differences in BDZ enhancement of GABA-stimulated Cl^- flux in the presence and absence of Zn^{2+} were comparable, the relative enhancement was greatly increased in the presence of Zn^{2+}. For example, in cortical microsacs without Zn^{2+} the flunitrazepam-induced increase in Cl^- flux evoked by 10 μM GABA of 3.4 nmol/mg of protein/3 sec represented 34% enhancement over base line, but in the presence of Zn^{2+} the absolute enhancement of 4.4 nmol/mg of protein/3 sec represented 163% potentiation. Similarly, in cer-

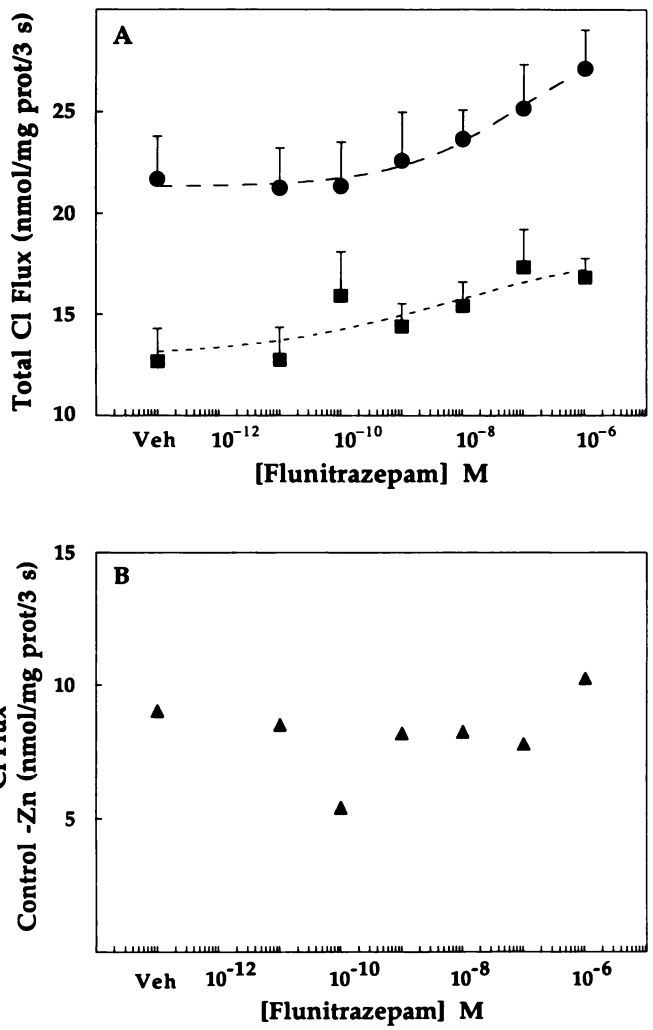

Fig. 3. A, Flunitrazepam concentration-response curves for the enhancement by 10 μM GABA of $^{36}Cl^-$ influx into cerebellar microsacs in the absence (●) and presence (■) of 100 μM Zn^{2+}. Total flux was calculated by subtracting only the filter blank from the data points. The *curves* were computed by fitting the data to eq. 1. B, The absolute difference between the data points in A is plotted against flunitrazepam concentration. *Veh*, vehicle.

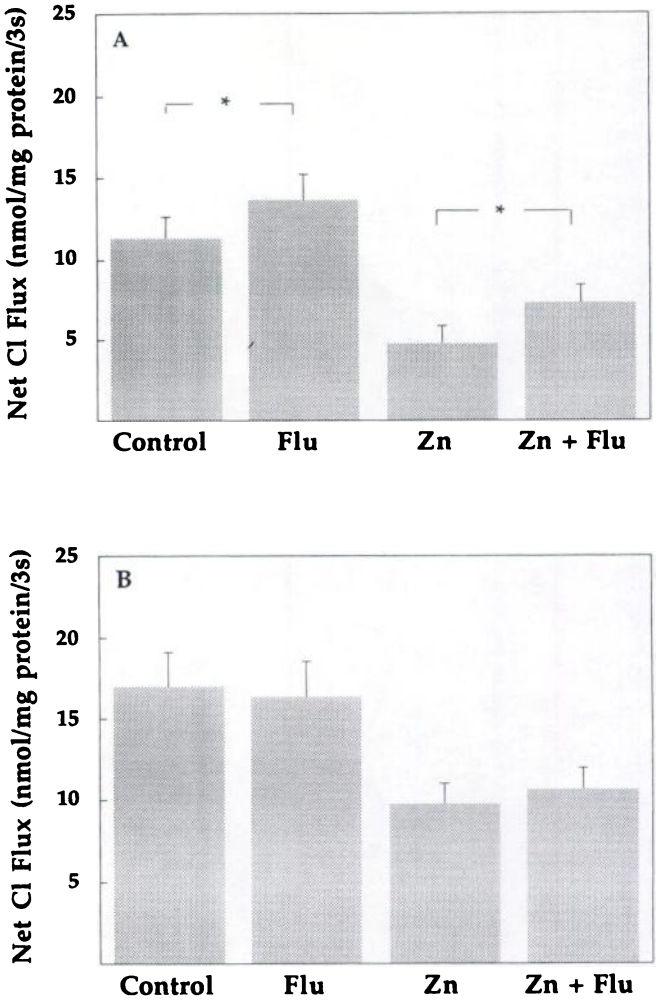

Fig. 4. Effect of Zn^{2+} on enhancement by 1 μM flunitrazepam (*Flu*) of Cl^- flux evoked by 10 μM GABA (A) and 100 μM GABA (B) in cerebellar microsacs. Data represent the mean ± standard error of 14 experiments. Flux is expressed as increase over Cl^- flux in the nominal absence of GABA. Statistical significance was tested using the ANOVA repeated-measures test. *, $p < 0.05$.

ebellar microsacs flunitrazepam caused only a 21% increase without Zn^{2+} but this increased to 54% in the presence of Zn^{2+}.

Effect of 100 μM Zn^{2+} on [^{3}H]Ro15–1788 binding. In cerebellar microsacs 100 μM Zn^{2+} increased the amount of specific binding of [^{3}H]Ro15–1788 by 34.0 ± 2.5%. However, the IC_{50} values for Ro15–1788 (0.8 nM) and flunitrazepam (4 nM) were unchanged.

Discussion

This study clearly shows that, in both adult rat cerebellum and cortex, there are certain populations of $GABA_A$ receptors that are sensitive to Zn^{2+} and insensitive to BDZ modulation, whereas, in contrast, the remaining GABA receptors that still function in the presence of Zn^{2+} are fully sensitive to BDZ enhancement. This result is consistent with the idea that Zn^{2+} blocks the functioning of only those $GABA_A$ receptor/Cl^- ionophores that lack a γ subunit (12, 13) and would therefore be insensitive to BDZ. The results obtained in this study also show that the proportion of Zn^{2+}-sensitive/BDZ-insensitive $GABA_A$ receptors composed of at least two populations is substantial, and they suggest that these sites play an important role in the functioning of the adult central nervous system.

In the presence of Zn^{2+}, flunitrazepam produces a greater relative degree of enhancement of Cl^- flux into cortical microsacs, compared with cerebellar microsacs. This observation indicates that the population of Zn^{2+}-insensitive/BDZ-sensitive $GABA_A$ receptor-ionophore complexes in cortical microsacs are more susceptible to potentiation by flunitrazepam. In cortex, 70% of the BDZ receptors have been characterized as type I receptors, whereas almost all receptors in the cerebellum are type I (25–27). The larger percentage of enhancement by flunitrazepam in cortical microsacs suggests that the Cl^- flux associated with non-type I BDZ receptors is potentiated to a greater extent than is flux through channels associated with type I BDZ receptors.

In general, BDZs preferentially enhance the effectiveness of low concentrations of GABA but do not influence its maximal effectiveness (28). This is in keeping with the ability of BDZs to increase the frequency of Cl^- channel opening (28). However, in the cortical microsacs with Zn^{2+} present, but not in cerebellar

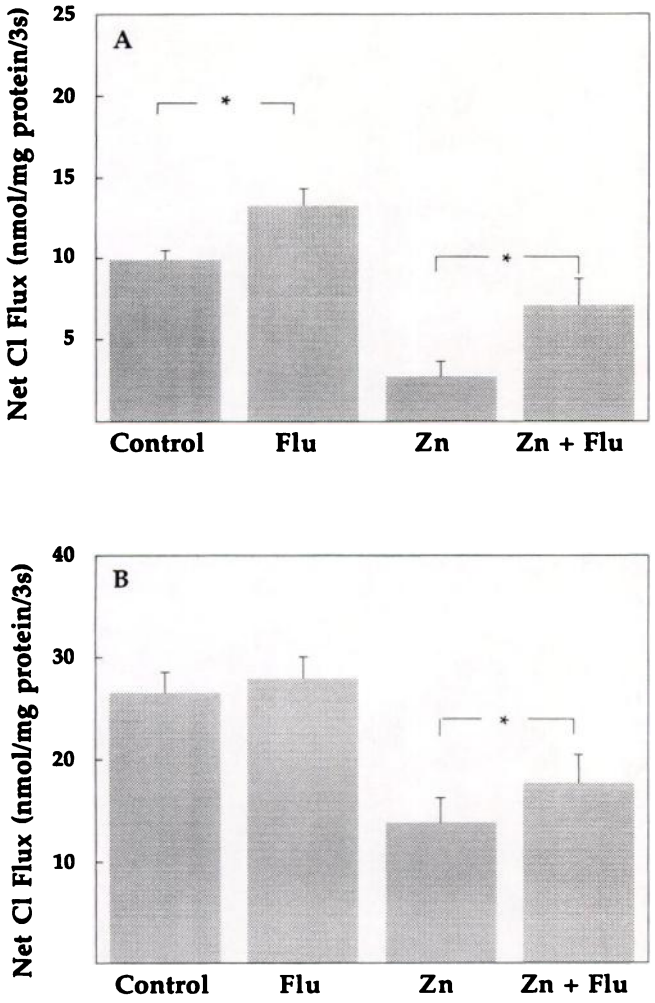

Fig. 5. Effects of Zn^{2+} on enhancement by 1 μM flunitrazepam (*Flu*) of Cl^- flux evoked by 10 μM GABA (A) and 100 μM GABA (B) in cortical microsacs. Data represent the mean ± standard error of nine experiments. Flux is expressed as increase over Cl^- flux in the nominal absence of GABA. Statistical significance was tested using the ANOVA repeated-measures test. *, $p < 0.05$.

TABLE 2

Effect of Zn^{2+} on the absolute enhancement of Cl^- flux produced by 1 μM flunitrazepam in cerebellar and cortical microsacs

The difference between the Cl^- flux produced by 10 μM and 100 μM GABA in the absence and presence of 1 μM flunitrazepam was calculated by subtraction of the values for individual experiments (nine experiments).

	Cl^- flux enhancement			
	10 μM GABA		100 μM GABA	
	Control	100 μM Zn^{2+}	Control	100 μM Zn^{2+}
	nmol/mg of protein/3 sec			
Cerebellum	2.4 ± 0.8	2.6 ± 0.9[a]	−0.6 ± 1.3	0.9 ± 0.9[a]
Cortex	3.4 ± 1.0	4.4 ± 1.6[a]	1.4 ± 1.2	3.9 ± 1.0[b]

[a] Not significant.
[b] $p < 0.05$.

microsacs, flunitrazepam produced a significant increase in Cl^- flux stimulated by 100 μM GABA, a nearly maximally effective concentration in this tissue.[1] This effect may reflect a difference in the characteristics of BDZ-sensitive GABA receptors in cortical and cerebellar tissues, because this effect was not observed in cerebellar microsacs. It does not appear that the maximal level of Cl^- flux into cortical microsacs was achieved, because barbiturates have been shown to substantially increase the plateau phase of the GABA concentration-response curve in this preparation (29, 30). However, the reasons for the enhanced BDZ sensitivity of cortical $GABA_A$ receptors, with respect to the Cl^- flux evoked by 100 μM GABA, when a population of $GABA_A$ receptors are blocked by Zn^{2+}, as observed in this study, are not yet understood. The cortical $GABA_A$ receptor-channel complex is also known to undergo desensitization, and the rate of desensitization is enhanced by increasing GABA concentrations and also by BDZs like flunitrazepam (31). The mechanism of desensitization is also not yet completely elucidated, but a role for phosphorylation of the receptor by protein kinase A under control of cAMP levels has been established (32–36). However, as yet there are no identified pathways that would explain the relationship between the rate of desensitization and GABA concentration or the presence of BDZs. Additional studies would be required to resolve this issue.

Although Zn^{2+} does not block BDZ-sensitive GABA receptors, there does seem to be some form of interaction between Zn^{2+} and these GABA receptors, because Zn^{2+} modestly increased [^{3}H]Ro15–1788 binding, an effect shared with other divalent cations (37). The trivalent cation lanthanum has recently been shown to increase the sensitivity of GABA for expressed receptors that contain the $\gamma 2$ subunit (38). It is conceivable that the effect of Zn^{2+} in increasing binding may occur by a similar mechanism.

These studies have established that there is a population of Zn^{2+}-sensitive/BDZ-insensitive $GABA_A$ receptors in adult brain that can be blocked by 100 μM Zn^{2+}. This finding has practical significance, because it can now be incorporated into more effective methods for evaluating BDZ modulation of the remaining population of Zn^{2+}-insensitive/BDZ-sensitive GABA receptors. However, the results still leave open the question of whether Zn^{2+} interaction with BDZ-insensitive $GABA_A$ receptors occurs in normal physiological functions. There is some evidence on both sides of this question. For example, there are high concentrations of Zn^{2+} stored in synaptic vesicles throughout the telencephalon (39) that can be released (40, 41). It has been estimated that, in the hippocampus, synaptic cleft concentrations of Zn^{2+} may reach 300 μM (42) and, therefore, could significantly inhibit any Zn^{2+}-sensitive GABA receptors in the area. However, our finding of Zn^{2+}-sensitive GABA receptors not only in cortex, an area rich in intravesicular Zn^{2+} (39), but also in cerebellum, an area where Zn^{2+} staining is localized to a distinct subset of mossy fiber glomeruli (43), would suggest that, although these receptors have the potential to be modulated by Zn^{2+}, this does not necessarily occur under physiological conditions.

Whether or not Zn^{2+} plays a role in modulating GABA receptors under physiological conditions, our finding that the population of Zn^{2+}-sensitive $GABA_A$ receptors are insensitive to BDZ and are a significant proportion of all such receptors can explain why many physiological functions are minimally affected by BDZs, even though $GABA_A$ receptors are ubiquitous in the brain and mediate most inhibitory neural activity.

Acknowledgments

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[1] Davies, M. F., P. A. Maguire, and G. H. Loew, unpublished observations.

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