Zinc and Zolpidem Modulate mIPSCs in Rat Neocortical Pyramidal Neurons

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DeFazio, Tony and John J. Hablitz. Zinc and zolpidem modulate mIPSCs in rat neocortical pyramidal neurons. J. Neurophysiol. 80: 1670–1677, 1998. Pharmacological modulation of γ-aminobutyric acid-A (GABA_A) receptors can provide important information on the types of subunits composing these receptors. In recombinant studies, zinc more potent inhibits αβ subunits compared with the αβγ combination, whereas modulation by nanomolar concentrations of the benzodiazepine type 1-selective agonist zolpidem is conferred by the α1β2γ2 subunit combination. We examined four properties of miniature inhibitory postsynaptic currents (mIPSCs) from identified neocortical pyramidal cells in rat brain slices: decay time constant, peak amplitude, rate of rise, and interevent interval. Exposure to 50 μM zinc reduced the decay time constant, peak amplitude, and rate of rise without effect on interevent interval. Zolpidem enhanced mIPSCs in a concentration-dependent manner. Both 20 and 100 nM zolpidem increased the decay time constants of mIPSCs. In some cells, both peak amplitude and rate of rise were also enhanced. All cells treated with zinc were also responsive to zolpidem. These results show that neocortical pyramidal cells have a population of GABA_A receptors sensitive to both zinc and zolpidem.

INTRODUCTION

Fast, chloride-dependent inhibitory synaptic transmission in the rat neocortex is mediated by γ-aminobutyric acid-A receptors (GABA_ARs) (Connors et al. 1988; Howe et al. 1987; Krnjevic and Schwartz 1967). GABA_ARs are the site of action of many convulsant and anticonvulsant drugs that interact with inhibitory synaptic mechanisms in the neocortex (Weiss and Hablitz 1984). Spontaneous inhibitory postsynaptic potentials are prominent in neocortical pyramidal cells (e.g., Luhmann and Prince 1991). Recent electrophysiological characterization of spontaneous and miniature inhibitory postsynaptic currents (mIPSCs) indicated that there is a tonic activation of GABA_ARs that may regulate neuronal excitability (Salin and Prince 1996; Xiang et al. 1998).

Molecular cloning studies identified ≈14 genes encoding GABA_AR subunits (Macdonald and Olsen 1994; Smith and Olsen 1995). Functional GABA_ARs of the central nervous system are presumed to be heteropentameric structures assembled from a combination of homologous subunits from several classes: α1–6, β1–4, γ1–4, δ, and ε (Chang et al. 1996; Nayeem et al. 1994; Whiting et al. 1997). The most common combination is thought to be α1βγ2, which makes up 43% of the GABA_ARs in the rat brain (McKernan and Whiting 1996). The subunit composition(s) of native GABA_ARs in neocortical pyramidal neurons is presently unknown, but messenger ribonucleic acids (mRNAs) for α1, β2, γ2, and δ are at least moderately expressed in adult rat neocortex in addition to weak signals for the α2, α3, α4, β3, and γ1 subunits (Laurie et al. 1992; Wisden et al. 1992). Subunit-specific antibodies show a similar pattern of expression, with diffuse but intense labeling of α1, β2/3, and γ2 subunits and moderate to weak labeling of α2, α3, α5, and δ subunits (Fritschy and Mohler 1995).

Because the subunit composition of the receptor can determine sensitivity to pharmacological agents that modulate the GABA_AR, it is possible that these agents could be used to identify the types of subunits composing native GABA_ARs. Two potentially useful agents are the imidazopyridine zolpidem, the triazolopyridazine CI 218,872, and the 1,4-benzodiazepine 2-oxo-quazepam have a greater affinity for the BZ1 receptor, whereas many of the other agonists for the BZ site lack specificity for the BZ1 and BZ2 sites (for review see Seighart 1995).

Single GABA_AR subunits or specific combinations were expressed in a number of recombinant expression systems. Detailed kinetic and pharmacological analyses assessed the role of the various subunits in determining sensitivity to BZ-related compounds and zinc. Recombinant systems expressing the α1 and γ2 subunits in combination with a variety of β subunits give rise to a BZ1-like receptor, whereas changing the α subunit subtype to α2, α3, or α5 gives rise to a BZ2-like pharmacology (Pritchett and Seeburg 1990; Pritchett et al. 1989a,b). The α6 subunit is expressed only in cerebellar granule cells and is not sensitive to most BZ agonists when expressed with β2 and γ2 subunits (Luddens et al. 1990). The γ subunit is also critical because BZ-related pharmacology is absent without it (Pritchett et al. 1989b). γ1 subunits are thought to be localized to glia and do not support BZ-like pharmacology. γ2 subunits are the most common in mammalian brain and in combination with the above-mentioned α subunits give rise to most of the high-affinity BZ receptors. γ3 subunits in combination with α1 or α5 subunits give rise zolpidem-insensitive receptors with high affinity for the compound CI 218,872 (for review see Luddens et al. 1995). Neither δ nor ε subunits support BZ efficacy in the absence of a γ subunit (Saxena and Mac-
TABLE 1.  Basic properties of miniature inhibitory postsynaptic currents

<table>
<thead>
<tr>
<th></th>
<th>Zinc Study (n = 6)</th>
<th>Zolpidem Study (n = 10)</th>
<th>Combined (n = 13)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Decay time constant, ms</td>
<td>17.9 ± 0.95</td>
<td>17.0 ± 1.2</td>
<td>17.7 ± 0.91</td>
</tr>
<tr>
<td>Rate of rise, pA/ms</td>
<td>66.5 ± 3.8</td>
<td>79.8 ± 4.9</td>
<td>70.8 ± 3.7</td>
</tr>
<tr>
<td>Peak amplitude, pA</td>
<td>−38.4 ± 3.0</td>
<td>−44.1 ± 2.1</td>
<td>−40.0 ± 2.2</td>
</tr>
<tr>
<td>Interevent interval, ms</td>
<td>594 ± 218</td>
<td>484 ± 66</td>
<td>585 ± 160</td>
</tr>
</tbody>
</table>

The values in this table represent means of control distributions averaged across cells in each study ± SE. Three cells of the zinc study were also used in the zolpidem study and were only counted once for the combined average. Number of cells in the Zinc Study was 6, in the Zolpidem Study was 10, and in Combined was 13.

Zinc sensitivity also depends on subunit composition. Recombinant GABA<sub>A</sub>Rs consisting of αβ subunits are very sensitive to zinc (IC<sub>50</sub> ~ 1 μM) via an apparent noncompetitive mechanism (Draguhn et al. 1990; Gingrich and Burkat 1998; Smart et al. 1991; Wooltorton et al. 1997). Zinc is a mixed antagonist of recombinant receptors containing α1β2γ2 subunits where it is less potent (IC<sub>50</sub> ~ 50 μM) (Chang et al. 1995; Gingrich and Burkat 1998). Both the αβγ and αβε receptors have a sensitivity to zinc intermediate to that of αβ and αβγ receptors (Krishek et al. 1998; Saxena and Macdonald 1994; Whiting et al. 1997). The α subunit subtype may also affect zinc sensitivity; maximal inhibition of the αβγ receptor with the α2 or α3 subunit is greater than α1-containing receptors (White and Gurley 1995), and zinc is much more potent at α6β3γ2 receptors than at α1β3γ2 receptors (Fisher and Macdonald 1997, 1998).

In an effort to pharmacologically identify the subunits mediating GABA<sub>A</sub>R-mediated inhibitory postsynaptic currents (IPSCs) in acute brain slices of the rat neocortex, we used the whole cell patch-clamp technique to measure the effects of zinc and zolpidem on miniature IPSCs. Some of these findings were presented in abstract form (DeFazio and Hablitz 1997).

METHODS

Slice preparation

Sprague Dawley rats (17–27 days old) were housed and handled according to approved guidelines. Rats were anesthetized with ketamine and decapitated. The brain was rapidly removed and submerged in ice-cold, oxygenated (95% O<sub>2</sub>, 5% CO<sub>2</sub>) low calcium saline consisting of (in mM) 125 NaCl, 3.5 KCl, 26 NaHCO<sub>3</sub>, 3.8 MgCl<sub>2</sub>, and 10 glucose. Coronal sections (300 μm) containing somatosensory cortex were cut with a Vibratome. Slices were stored in saline consisting of (in mM) 125 NaCl, 3.5 KCl, 26 NaHCO<sub>3</sub>, 2.5 CaCl<sub>2</sub>, 1.3 MgCl<sub>2</sub>, and 10 glucose bubbled with 95% O<sub>2</sub>, 5% CO<sub>2</sub>. Recordings were begun after a stabilization period of ≥1 h.

Recording

Individual slices were transferred to a recording chamber mounted on the stage of a Axioskop FS microscope equipped for infrared visualization of cells. Whole cell voltage-clamp recordings were obtained from layers II/III and V neocortical pyramidal cells with patch electrodes prepared from Garner KG-33 glass. The electrodes had resistances of 2–4 MΩ when filled with an internal solution consisting of (in mM) 140 CsCl, 10 N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid, 1 ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid, 0.1 CaCl<sub>2</sub>, 4 MgATP, and 0.4 NaGTP. The bathing solution consisted of the storage saline listed above with the addition of the sodium channel blocker (tetrodotoxin, 0.5 μM) and the excitatory amino acid blockers D(-)-2-amino-5-phosphonovaleric acid (APV) and 6-cyano-7-nitroquinoxaline-2,3-dione (0.5, 20, and 10 μM, respectively). Membrane current was amplified with an Axopatch-1B amplifier. Signals were filtered at 2 kHz and digitized at 5 kHz in 40- to 60-s duration epochs. Series resistance and input resistance were monitored regularly during recording. Cells with series resistance >20 MΩ or significant changes in series resistance during the experiment were not used. The mean input resistance was 215 ± 20 MΩ and ranged from 144–333 MΩ.

Zolpidem and ZnCl<sub>2</sub> were dissolved in 100% ethanol to make concentrated stock solutions weekly and were stored at −20°C. Control experiments with ethanol alone showed no effect on any of the mIPSC parameters used in this study. All compounds were obtained from Sigma with the exception of zolpidem (RBI).

Data analysis

pClamp data files were imported into CDR for event detection and subsequently exported into SCAN for analysis (CDR and SCAN programs courtesy of J. Dempster). Decay time constants, rate of rise, peak amplitudes, and interevent intervals for single IPSCs were measured with SCAN. Rate of rise was measured as the maximum rate of rise ±10–90% of the peak height. Although
a fraction of mIPSCs were better fit by double exponentials, for ease of analysis single exponential fits (20- to 30-ms fit window, forced to a zero baseline) were used throughout this study. Similar results were obtained by using the full width at half-maximum to measure mIPSC duration. Averaged mIPSCs were generated after aligning events on the rising phase. Cumulative probability plots (Van der Kloot 1989) for each parameter were generated from ≥50 events (usually ~100) for each control and drug application. These plots were generated by sorting the values for a given parameter in ascending order and then assigning a probability between 0 and 1 based on the number of events preceding the event normalized by the total number of events in the distribution. Distributions were analyzed with the Kolmogorov-Smirnov test (StatMost data analysis software, DataMost) and a $P < 0.01$ significance level for distributions with $<100$ events and $P < 0.05$ otherwise. Plots of distributions and averaged traces contained data from individual cells. Summary bar graphs reflect the average of the percent change relative to control of individual cells.

**RESULTS**

**Properties of mIPSCs**

Under the recording conditions employed, pharmacologically isolated mIPSCs were recorded as inward currents at a holding potential of −60 mV. As described previously (Salin and Prince 1996; Xiang et al. 1998; Zhou and Hablitz 1997), mIPSCs had rapid rise times and decayed with an exponential time course. Table 1 lists average decay time constants ($\tau_{\text{decay}}$), rates of rise, peak amplitude, and interval event under control conditions. The cells common to both the zinc and zolpidem groups were only counted once for the combined group. No significant differences were observed between the zinc and zolpidem groups, the mean CV for the peak amplitude (59.5 ± 2.5%, $n = 13$) was comparable with that observed in cerebellar stellate cells (Nusser et al. 1997) and cultured retinal amacrine cells (Frerking et al. 1997).

Zinc reduces amplitude, rate of rise, and decay time constant

Figure 1 shows averaged mIPSCs before and after bath application of 50 $\mu$M zinc. The primary effects of zinc were a reversible reduction in peak amplitude and $\tau_{\text{decay}}$. By normalizing the peak amplitudes of the averaged traces, the reversible decrease in $\tau_{\text{decay}}$ is readily apparent (Fig. 1B). Probability distributions for $\tau_{\text{decay}}$, peak amplitude, rate of rise, and interevent interval are shown in Fig. 2. The $\tau_{\text{decay}}$ was decreased in the presence of 50 $\mu$M zinc, as indicated by the leftward shift of the distribution ($P < 0.01$, Kolmogorov-Smirnov test). The effects of zinc on peak and rate of rise were more profound on larger events, possibly because of the smaller events falling below the threshold for detection.
The effect of 100 nM zolpidem on rate of rise and peak amplitude were reduced by 27.5% and 28.1% respectively. The slight increase in interevent interval was observed (Fig. 2D). No significant effect on interevent interval was observed (Fig. 5D).

Figure 6 summarizes the effects of zolpidem on mIPSCs. In every cell exposed to either 20 nM (n = 9) or 100 nM (n = 5) zolpidem, a significant enhancement of $\tau_{\text{decay}}$ was observed. On average this resulted in a 19.5 ± 2.4 and 27.2 ± 4.8% enhancement by 20 and 100 nM zolpidem, respectively. The large error bars for rate of rise and amplitude reflect the fact that only three of nine cells exposed to 20 nM zolpidem and two of five cells exposed to 100 nM zolpidem showed a significant effect on these parameters. The slight decrease in interevent interval may be the result of an increase in the number of events detected at threshold caused by amplitude enhancement by zolpidem.

**Neocortical pyramidal cells are responsive to both zinc and zolpidem**

A subset of cells tested with zinc was also exposed to zolpidem and was found to be sensitive to both compounds (n = 3). An example of the response of a zinc-sensitive cell to 20 nM zolpidem is shown in Fig. 7. Zolpidem (20 nM) significantly enhanced the mIPSC $\tau_{\text{decay}}$, as indicated by the rightward shift of the distribution of $\tau_{\text{decay}}$ in Fig. 7A (P < 0.001, Kolmogorov-Smirnov test). The minor enhancement of peak amplitude apparent in the averaged traces (Fig. 7B1) was not significant when the distributions of peak amplitudes were compared with the Kolmogorov-Smirnov test. Normalized traces shown in Fig. 7B2 illustrate the reversible increase in $\tau_{\text{decay}}$ in the presence 20 nM zolpidem.

**Zolpidem enhances decay time constant**

In the cell shown in Fig. 4A, bath applications of 20 and 100 nM zolpidem significantly enhanced the peak amplitude of averaged mIPSCs in a concentration-dependent fashion. The changes in $\tau_{\text{decay}}$ became apparent when the amplitudes of the averaged mIPSCs were normalized (Fig. 4B). The effect of 100 nM zolpidem on $\tau_{\text{decay}}$ was only partially reversed after 5 min of wash. Figure 5 shows cumulative probability plots of the data from the cell shown in Fig. 4. Both concentrations of zolpidem significantly enhanced $\tau_{\text{decay}}$. The effect of 100 nM zolpidem persisted after >5 min wash (Fig. 5A). Although in other experiments both the effects of zinc and 20 nM zolpidem could be reversed with 5 min wash, the effect of 100 nM zolpidem on $\tau_{\text{decay}}$ was not completely reversed in any of the cells with >5 min wash (wash distribution significantly greater than control, n = 4). This lack of reversibility may be due to the lipophilic nature of zolpidem, which would make it difficult to wash out of the brain slice.

Twenty and 100 nM zolpidem significantly and reversibly enhanced IPSC amplitude in this cell (Fig. 5B). Rate of rise was reversibly enhanced by 100 nM zolpidem (Fig. 5C). No significant effect on interevent interval was observed (Fig. 5D).
FIG. 5. Effects of zolpidem on individual events are shown in cumulative probability plots. A: modest enhancement of the decay kinetics by 20 nM zolpidem is reflected in a small but significant rightward shift in the distribution of $\tau_{\text{decay}}$; 100 nM zolpidem produced a much larger rightward shift, and, consistent with the effects on averaged mIPSCs, the distribution of $\tau_{\text{decay}}$ after 5-min wash was significantly greater than the control distribution. B and C: peak amplitude and rate of rise distributions also reflect enhancement by both concentrations of zolpidem, although the effect on amplitude and rate was reversible. D: no significant effects on the interevent interval distributions were observed. The number of events for each distribution are the same as in Fig. 4. As in Fig. 2, boxes associated with each plot show the level of significance of the differences between the distributions listed in rows and columns: $\neg$, not significant; * $P < 0.05$; and ** $P < 0.01$.

DISCUSSION

The results of this study demonstrate that GABA$_A$R-mediated mIPSCs in rat neocortical pyramidal cells can be modulated by both zinc and zolpidem. These agents had opposing effects on mIPSC $\tau_{\text{decay}}$ distributions; zinc shifted the distribution of time constants to smaller values, whereas zolpidem shifted the distribution to larger values. In contrast to the effects of ethanol on evoked hippocampal IPSCs (Weiner et al. 1997) and the BZ receptor agonist flurazepam in cerebellar stellate cells (Nusser et al. 1997), differential modulation of subpopulations of mIPSCs by zinc or zolpidem was not apparent in the decay time constant distributions (e.g., Figs. 2 and 5). This suggests that most of the receptors on rat neocortical pyramidal cells are responsive to both zinc and zolpidem (e.g., Fig. 7).

Properties of mIPSCs in neocortical pyramidal cells

Previous studies examined the kinetic properties of mIPSCs in neocortical interneurons and pyramidal cells (Salin and Prince 1996; Xiang et al. 1998; Zhou and Hablitz 1997). Similar to previous studies in neocortex and other central neurons, mIPSC amplitudes were highly variable, ranging from 10 to 200 pA. Amplitude histograms were generally not well fit with single or sums of multiple Gaussian functions (data not shown). As measured by the coefficient of variance, amplitude variability was similar to that observed in cerebellar stellate cells and retinal amacrine cells (>50%) (Frerking et al. 1997; Nusser et al. 1997). The origins of this variability and non-Gaussian distribution are not well understood. Both pre- and postsynaptic mechanisms were hypothesized to underlie this phenomenon. These include variability in the number of receptors at postsynaptic sites (Nusser et al. 1997) or variability in the amount of transmitter released from vesicles (Frerking et al. 1995).

Zinc and zolpidem have opposite effects on mIPSCs

The current results demonstrate that GABA$_A$Rs on rat neocortical pyramidal cells are sensitive to both zolpidem and zinc. Zinc (50 $\mu$M) reduced the rate of rise, amplitude, and $\tau_{\text{decay}}$. These properties are consistent with a reduction in the affinity of the receptor for GABA, possibly via a mechanism of “mixed antagonism” similar to that observed for the recombinant $\rho$ GABA receptor (Chang et al. 1995) or $\alpha1\beta2$ receptors (Gingrich and Burkat 1998; Krishek et al. 1998). Contrary to previous reports in the hippocampus (Buhl et al. 1996), no presynaptic effect of zinc on mIPSC frequency was observed.

Zolpidem consistently enhanced the $\tau_{\text{decay}}$ and in some cells also increased the amplitude and rate of rise of mIPSCs.
Zinc and Zolpidem Modulate mIPSCs

suggests that this is not the case for native neocortical pyramidal cell GABA_A Rs.

In cerebellar stellate cells, a subpopulation of mIPSCs with larger amplitudes and greater sensitivity to the BZ agonist flurazepam was observed in the probability distribution (Nusser et al. 1997). In this study, the effects of both zinc and zolpidem on amplitude and rate of rise were greater on larger, faster events, whereas the τ_decay distributions tended to reflect shifts in the entire distribution. These inconsistent effects could possibly be explained by variation in the degree of saturation at different release sites. For the case of zolpidem, amplitude modulation via a change in affinity for GABA would not be observed at saturated release sites. If the smaller mIPSCs were due to release sites that were saturated because of the presence of fewer receptors, no amplitude modulation would occur. However, the decay time constant would be enhanced at both saturated and nonsaturated release sites. A similar argument can be made for zinc, assuming a zinc-mediated decrease in affinity for GABA. Another possibility is that the smallest events were lost in the baseline noise, which would force all the amplitude distributions to start at roughly the same value.

Implications for the pharmacological identification of native receptors

All cells exposed to zinc and zolpidem exhibited sensitivity to both agents (e.g., Fig. 7). Enhancement of the mIPSC τ_decay distribution in response to 20 nM zolpidem indicates the presence of the high-affinity BZ1 receptor thought to be conferred by an α1β2γ2 subunit combination. This is further supported by a moderate sensitivity to zinc. Previous studies with native receptors in other brain regions revealed sensitivity to both BZ agonists and moderate concentrations of zinc (Celentano et al. 1991; Legendre and Westbrook 1991; Smart 1992). Alterations in zinc and BZ sensitivity were

Zolpidem and other BZs are thought to modulate GABA_A Rs by increasing the affinity for GABA and increasing the frequency of channel opening (Rogers et al. 1994). Under nonsaturating conditions, this modulation would increase the amplitude of the GABA response (Frerking et al. 1995; Lavoie and Twyman 1996; Poncer et al. 1996). The observation that zolpidem increased mIPSC amplitude in some cells indicates that rat neocortical neurons may have postsynaptic sites that are not saturated by quantal release of GABA (Frerking et al. 1995; Nusser et al. 1997). BZs may also increase single channel conductance (Eghbali et al. 1997), although the inconsistent effect of zolpidem on amplitude

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**Figure 6.** Summary of the effects of zolpidem. The largest effect of zolpidem was on the enhancement of the τ_decay distribution, 19.0 ± 2.7% and 27.4 ± 4.8% for 20 nM and 100 nM zolpidem, respectively. Lack of effect on rate of rise and peak amplitude on most cells is reflected in the SE of the average increase.

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**Figure 7.** Cells responsive to zinc were also responsive to zolpidem. **A:** cell from Figs. 1 and 2 was also sensitive to 20 nM zolpidem. The distribution of τ_decay was reversibly shifted to the right. The zolpidem distribution was significantly greater than either control or wash, P < 0.01. Control and wash distributions were not significantly different. **B:** averaged traces (B1) and normalized (B2) averages are shown to illustrate the effects on τ_decay. Numbers of events for each distribution and average were 128, 126, and 113 for control, 20 nM zolpidem, and wash, respectively.
observed in several animal models of epilepsy (Buhl et al. 1996; Gibbs et al. 1997; Kapur and Macdonald 1997).

However, more complicated scenarios cannot be excluded. In situ hybridization revealed mRNAs for α1, α2, α3, α4, β2, β3, γ1, γ2, and δ subunits in layer II/III of adult rat neocortex (reviewed in Wisden et al. 1995). Similar distributions of subunits were identified with antibodies (Fritschy and Mohler 1995). The possibility that a combination of different subunit subtypes within the heteropentameric GABA A R complex could also give rise to this dual sensitivity cannot be ignored because the pharmacology of multiple combinations of α subunits has not been thoroughly studied in recombinant systems. The δ subunit also could enhance the zinc sensitivity of αβγ receptors (Saxena and Macdonald 1994).

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