Effect of Zolpidem on Miniature IPSCs and Occupancy of Postsynaptic GABA<sub>A</sub> Receptors in Central Synapses

David Perrais and Nicole Ropert

Institut Alfred Fessard, Centre National de la Recherche Scientifique UPR 2212, Gif sur Yvette, France

GABA<sub>A</sub>-mediated miniature IPSCs (mIPSCs) were recorded from layer V pyramidal neurons of the visual cortex using whole-cell patch-clamp recording in rat brain slices. At room temperature, the benzodiazepine site agonist zolpidem enhanced both the amplitude (to 138 ± 26% of control value at 10 μM) and the duration (163 ± 14%) of mIPSCs. The enhancement of mIPSC amplitude was not caused by an increase of the single-channel conductance of the postsynaptic receptors, as determined by peak-scaled non-stationary fluctuation analysis of mIPSCs. The effect of zolpidem on fast, synaptic-like (1 msec duration) applications of GABA to outside-out patches was also investigated. The EC<sub>50</sub> for fast GABA applications was 310 μM. In patches, zolpidem enhanced the amplitude of currents elicited by subsaturating GABA applications (100–300 μM) but not by saturating applications (10 mM). The increase of mIPSC amplitude by zolpidem provides evidence that the GABA<sub>A</sub> receptors are not saturated during miniature synaptic transmission in the recorded cells. By comparing the facilitation induced by 1 μM zolpidem on outside-out patches and mIPSCs, we estimated the concentration of GABA seen by the postsynaptic GABA<sub>A</sub> receptors to be ~300 μM after single vesicle release. We have estimated a similar degree of receptor occupancy at room and physiological temperature. However, at 35°C, zolpidem did not enhance the amplitude of mIPSCs or of subsaturating GABA applications on patches, implying that, in these neurons, zolpidem cannot be used to probe the degree of receptor occupancy at physiological temperature.

Key words: benzodiazepines; zolpidem; γ-aminobutyric acid type A receptors; miniature inhibitory postsynaptic currents; synaptic transmission

In the Central Nervous System, fast inhibitory synaptic transmission is primarily mediated by GABA acting on GABA<sub>A</sub> receptors. They bear various modulatory sites, among them the benzodiazepine (BZD) site (MacDonald and Olsen, 1994). The effects of BZD agonists on currents elicited by GABA to native or recombinant receptors (for review, see MacDonald and Olsen, 1994; Lavoie and Twyman, 1996; Mellor and Randall, 1997) are consistent with a change in the binding kinetics of GABA as well as desensitization kinetics of the receptor. This makes BZD agonists tools of choice to study the parameters of GABA<sub>A</sub> receptor activation during synaptic transmission (De Koninck and Mody, 1994; Frerking et al., 1995).

The high concentration of glutamate estimated in the synaptic cleft at excitatory synapses (Clements et al., 1992) has led to the hypothesis that receptor saturation occurs during a synaptic event. This assumption is consistent with the observation of peaky amplitude distributions of the evoked postsynaptic currents (Edwards et al., 1990) that result from the summation of elementary events, quanta, with a low coefficient of variation (CV). Given the low number of receptors activated during a miniature synaptic event (Edwards et al., 1990; Ropert et al., 1990), a low CV can be obtained if the channel open probability is very high at the peak of the synaptic current (Jonas et al., 1993), implying that the degree of occupancy of postsynaptic receptors is high during synaptic transmission.

Received April 30, 1998; revised Oct. 22, 1998; accepted Oct. 29, 1998.

We thank Drs. F. Sladecek and F. Le Bouffant for their support with some of the equipment, and G. Sadoc for help with the acquisition and analysis software. We also thank Drs. B. Barbour and M. Häusser, and N. Gazères for useful discussions.

MATERIALS AND METHODS

Brain slice preparation. Slices were prepared from young male Wistar rats (mean 17 d old; range, 15–25 d old). The animals were anesthetized with...
sodium pentobarbital and decapitated. The brain was rapidly removed and submerged in oxygenated (5% CO₂, 95% O₂) cold artificial CSF (ACSF) for dissection of the occipital cortex. Slices (300 μm thickness) were cut in the sagittal plane using a vibratome (DTK-1000, DSK) and maintained at a temperature of 35°C for at least 1 hr before recording.

**Electrophysiology and data analysis.** The neurons were identified using an upright microscope (Axioskop, Zeiss) with Nomarski optics and an infrared video camera (Newvicon, Hamamatsu) as reported previously (Stuart et al., 1993). Most of the recordings were made at room temperature (22–25°C) from slices kept under constant (2 mL/min) ACSF perfusion. For the experiments of Figures 8 and 9, slices were recorded at 35°C. The ACSF was heated before entering the recording chamber. For outside-out patch recordings at 35°C, the application pipette was dipped into the bath along 5 mm, and thus the flowing solutions were heated to 35°C (Tonegawa et al., 1993). The temperature was measured by a thermal probe before and after each experiment.

Recording pipettes were made using cleaned and sterilized borosilicate glass. Their typical resistance was 1–2 MΩ for whole-cell recordings and 2–8 MΩ for outside-out somatic patch recordings. The pipettes were coated with beeswax. Recordings were performed using a patch-clamp amplifier (Axopatch 200A, Axon Instruments). During recording, the stability of the series resistance, between 5 and 15 MΩ, was checked using a +2 or +5 V voltage step applied every 20 sec, and the recording was discarded if it increased by >10%. Evoked activity was stored on a computer online. Spontaneous synaptic activity was filtered at 2 kHz and stored on digital tape recorder (DTR-1202, 48 kHz sampling rate, Biologic) for subsequent analysis. The data were acquired using a Digidata 1200 board (Axon Instruments) and analyzed using programmable software (Acquis 1, Biologic).

Spontaneous synaptic activity during periods of 1–3 min was digitized at 20 kHz. Between 200 and 1500 synaptic events per period were detected using a threshold crossing of the derivative with parameters set for each cell and kept constant for the whole session. The events detected were then visually inspected to remove electrical artifacts. Their peak amplitude and 10–90% rise time were measured. The decay phase of individual events (with no superimposition) could be fitted by one or more exponentials. Because the changes observed during zolpidem application did not consistently affect one component in particular, the description of mIPSCs was quantified by calculating an estimation of the time constant of decay (τᵣₖ) without any assumption on the number of decay components:

\[ \tau_r = \int I(t) dt / A, \]

where \( I \) is the current and \( A \) is the peak amplitude of the mIPSC. The integral is taken between the peak of the IPSC and the return to baseline. If one attempts to fit the decay by a sum of exponentials \( I(t) \sim \sum_A \exp(-t/\tau_i) \), then \( \tau_r \sim \sum_A \exp(-t/\tau_i) dt / A \) = \( \sum_A \tau_i / A \), which corresponds to the mean of the decay time constants used for the fit. The estimation of the duration \( \tau_r \) (term used in the rest of this paper) of the mIPSCs using this procedure and a classical fit with exponential functions gave similar results (see Fig. 1B).

The zolpidem concentration increase of mIPSC duration graphs (see Fig. 3) were fitted with the following equation:

\[ \tau_r/\tau_{r,control}(Z) = \max(1 + (EC_{50}/Z)^n), \]

where \( Z \) is the concentration of zolpidem, \( \tau_{r,control} \) is the duration of mIPSCs without zolpidem, \( \max \) is the maximal relative increase of the duration, \( EC_{50} \) is the half-maximal effect concentration, and \( h \) is the Hill coefficient. For each concentration, the stationarity of the mIPSC parameters was ascertained. Moreover, no change of the amplitude or the duration of mIPSCs was seen in control conditions over a period of 30 min, which exceeds the duration of the recordings necessary to test the effects of zolpidem.

Non-stationary fluctuation analysis (NSFA) was performed on currents elicited by fast applications of saturating concentrations of GABA on outside-out patches as described previously (Jonas et al., 1993). Series of 15–40 applications with stable maximal amplitude and duration were averaged. For each individual trace, the variance around the mean, the amplitude of the baseline noise, was computed for regularly spaced time intervals. For each interval, the corresponding mean current was measured, and the relation between the mean current \( I \) and the variance \( \sigma^2 \), minus the variance of the recording noise \( \sigma_{baseline}^2 \), was drawn. These two parameters can be decomposed as \( I = NP(I) \) and \( \sigma^2 = \sigma_{baseline}^2 = NP(I)(1 - P(I))^2 \), where \( N \) is the number of channels open at the peak of the current, \( P(I) \) is the open probability of channels, and \( i \) is the current carried by a single open channel. From these expressions a parabolic curve was fitted with the equation \( \sigma^2 = \sigma_{baseline}^2 = d - I^2/N \), giving \( i \) and \( N \). The maximal open probability of the channels was also calculated with \( P_{P,max} = 1 - (\rho_{peak} - \sigma_{baseline})/I_{peak} \), where \( \rho_{peak} \) and \( I_{peak} \) are the variance and the average of the current at its peak, respectively.

Peak-scaled NSFA was also performed on mIPSCs to estimate \( i \) (Traynelis et al., 1993; De Koninck and Mody, 1994; Silver et al., 1996). In cells where the mIPSC frequency was low enough, 30–100 mIPSCs were selected, with no overlap with other minis. The procedure was the same as for NSFA, except that the average of these mIPSCs was scaled to each individual mini before computing the variance. Therefore, the relation between \( I \) and \( \sigma^2 \) becomes \( \sigma^2 = \rho_{baseline}^2 + d - I^2/N_p \), where \( N_p \) is the number of channels open at the peak of the current.

All results are given as mean ± SD. The variability was measured by the CV, which is the ratio of the SD to the mean. The large sample approximation of the Kolmogorov–Smirnoff test (KS test) was used to compare the distributions of the mIPSCs. The paired or unpaired Student’s two-tailed \( t \) test was used to examine the level of significance of the results.

The extracellular standard ACSF contained (in mm): NaCl 126, KCl 1.5, KH₂PO₄ 1.25, MgSO₄ 1.5, CaCl₂ 2, NaHCO₃ 26, and glucose 10. GABAₐ- mediated mIPSCs were recorded in the presence of 6-cyano-7-nitroquinolxaline-2,3-dione (CNQX, 10 μM; Tocris) and d(-)-2-amino-5-phosphonopentoic acid (APV, 50 μM; Tocris) to block non-NMDA- and NMDA-mediated glutamatergic synaptic currents, and tetrodotoxin (TTX) to block action potentials (1 μM; Sigma, Janssen, or Latoxan). The mIPSCs or the GABA-evoked currents in outside-out patches were blocked by bicuculline methiodide (10 μM; Sigma) or picrotoxin (100 μM; Sigma). Zolpidem was a gift from Synthelabo Recherche, and flumazenil (Ro 15-788) was a gift from R. Corradetti (University of Firenze). Zolpidem was dissolved in water in stock solutions (5 mM), and flumazenil was dissolved in DMSO. The final fraction of DMSO was 0.2%, which had no effect on mIPSCs (n = 2) or on the effect of zolpidem (n = 4). In our preparation, the recovery after an application of zolpidem was not complete after 30 min wash out; therefore we took a new slice after each zolpidem inlay.

Intrappetite solutions for whole-cell recording contained (in mm): CsCl 140, HEPES 10, MgCl₂ 3, EGTA 0.5, pH 7.3, 280 mOsm. For outside-out patches and some whole-cell recordings, intrappetite solutions contained (in mm): CsCl 120, HEPES 10, ATP 4, GTP 0.5, MgCl₂ 2, EGTA 10, pH 7.3, 280 mOsm. Potentials were corrected for a −4 mV junction potential. Because no differences were seen between the recordings obtained with both intracellular solutions, the results were pooled together.

Fast application of GABA was performed on outside-out patches as described previously (Colquhoun et al., 1992). The control solution contained (in mm): NaCl 140, CsCl 2, KCl 1.5, MgCl₂ 1, HEPES 10, adjusted to pH 7.4. In the GABA-containing solution, we added 30 mM sucrose to visualize the interface between the control and the GABA solutions, and 10 mM NaCl to measure the 10–90% exchange time between the control and the agonist solutions after blowing out the patch, which was typically 0.2 msec (see Fig. 5). Applications were made every 10 sec to avoid desensitization of the GABAₐ receptors. Usually, the responses during the first few GABA applications tended to diminish before reaching a stationary level. This initial amplitude decrease did not seem to be attributable to cumulative desensitization of the receptors, because it was not dependent on the application frequency and was not reversed if the application was stopped. Then the response could remain stable up to 20 min. Up to four different solutions could be applied in each barrel by switching the perfusion tubes with a valve. The exchange time between the two solutions was ~30 sec. When the effect of bicuculline or zolpidem was tested on the response to the application of GABA, both control and GABA solutions contained the modulators.

**RESULTS**

**Description of mIPSCs**

Spontaneous GABAₐ-mediated miniature postsynaptic currents were recorded in layer V cortical pyramidal neurons in the presence of 10 μM CNQX, 50 μM APV, and 1 μM TTX at a holding potential of ~70 mV. The distributions of time intervals between events were fitted by a single exponential, as expected for a
Effect of zolpidem on mIPSCs

The effect of the BZD agonist zolpidem (10 μM) on mIPSCs was studied in layer V pyramidal cells (n = 12). It did not change the frequency of events (5.7 ± 3.0 Hz in control vs 6.1 ± 3.5 Hz in zolpidem; p > 0.05, paired t-test), consistent with the purely postsynaptic actions ascribed to this compound (Fig. 2C). Moreover, the input resistance of the cells and the noise level of the recordings were not changed by zolpidem. The observed effects are thus presumably caused exclusively by the binding of zolpidem to postsynaptic GABA_A receptors.

Zolpidem applied in the bath enhanced significantly both the duration (163 ± 14% of control; p < 0.001) and the amplitude (138 ± 26% of control; p < 0.005) of events (Fig. 2B). The amplitude distributions in control and in 10 μM zolpidem were significantly different (KS test; p < 0.001) in 10 of 12 pyramidal cells, and the distributions of durations were significantly different in the 12 cells (KS test; p < 0.001). Zolpidem is among the most selective known BZD agonists: three types of GABA_A receptors with high, intermediate, or low affinity for zolpidem can be differentiated (Lüddens et al., 1995). To test whether the zolpidem-induced increases in amplitude and duration were uniform, we compared the mIPSC distributions with and without zolpidem. We normalized the control distributions by a scaling factor equal to the ratio of the amplitude (or duration) in zolpidem and in control. These two distributions were not significantly different (KS test; p > 0.05) in the 12 pyramidal cells tested (Fig. 2C); therefore, the hypothesis of a non-uniform population of GABAergic synapses with distinct GABA_A receptor subtypes activated in the presence of TTX is not supported by our data.

Even if all of the postsynaptic receptor clusters, active during TTX application, are equally affected by zolpidem, not all of the receptors in a synapse are necessarily equally sensitive to this compound. To test the intrasynaptic heterogeneity of GABA_A receptors underlying mIPSCs in these cells, we looked at the effect of several concentrations of zolpidem (Fig. 3). The potentiation of the duration and amplitude were approximately parallel at low concentrations (<10 μM), but the effect on the amplitude decreased at the highest concentration tested (100 μM). In contrast, the dose–response curve for the duration could be fitted by the logistic equation (see Materials and Methods), and the calculated EC_{50}, Hill coefficient, and maximal effect were 5.8 μM, 0.36, and 221% of control duration, respectively. The effect of zolpidem (1 μM) was reversed by the BZD antagonist flumazenil (10 μM): the mean amplitude of mIPSCs returned to 94 ± 3% of control (n = 5), and their duration returned to 104 ± 5% of control (Fig. 3C).

It should be noted that the mIPSCs recorded are filtered to some extent, compared with the synaptic conductance (Llano et al., 1991; Jonas et al., 1993). Therefore, we examined whether a lengthening of the synaptic current at its source could induce a significant increase in the peak amplitude of the recorded current caused by filtering. The filter (cell and recording system) can be modeled in a first approximation as a low-pass capacitive filter, with a time constant T. Because there is no correlation between the amplitude, rise time, and duration of mIPSCs (Fig. 1C), the
events are presumably filtered to the same extent. We assumed a current source at the synapse $I_s(t)$ with an instantaneous rise and an exponential decay $\tau$ and calculated how this current amplitude is changed by filtering. The current source is $I_s(t) = I_s^0 \exp(-t/\tau)$ for $t \geq 0$ and $I_s(t) = 0$ for $t < 0$. The current recorded at the soma is $I(t) = I_s^0 (\tau/(\tau - T)) \exp(-t/\tau) - \exp(-t/T)$ for $t \geq 0$ (Llano et al., 1991). The time-to-peak of the current recorded at the soma is $t_p = \tau [T/(\tau - T)]\ln(\tau/T)$, its amplitude is $A(t_p) = I_s^0 \exp(-t_p/\tau)$, and its estimated time constant (see Materials and Methods) is $\tau_e = \tau [I(t_p) = \tau \exp(t_p/\tau)$. The highest rise time and the lowest duration of the recorded mIPSCs give values for events that are the most sensitive to filtering. They were chosen to estimate $T$ and $\tau$. The highest $10$–$90\%$ rise time of the mIPSCs (1.1 msec) gives a value of $t_p$ equal to 1.5 msec. From this value and the fastest decay of the mIPSCs (10.8 msec), we obtain a value of $\tau$ equal to 9 msec and a value of $T$ equal to 0.5 msec. In these conditions, when $\tau$ is doubled, which is more than the change in the mIPSC duration observed in 10 $\mu$M zolpidem (163% of the control value), the recorded mIPSC amplitude would be $107\%$ of the control amplitude, far lower than the value found (Fig. 3). Thus the increase of the amplitude of the recorded mIPSCs during zolpidem application is most likely mainly caused by an increase of the current source amplitude.

A postsynaptic current is attributable to the binding of the neurotransmitter and the activation of $N_\alpha$ independent channels (Edmonds et al., 1995). The opening probability of a channel that has bound the neurotransmitter is a function of time, termed $P_o(t)$, and therefore the postsynaptic current can be decomposed

---

**Figure 2.** Effects of bath application of 10 $\mu$M zolpidem on the mIPSCs recorded in a layer V cortical pyramidal cell. A. Recordings of miniature activity before and during the application of 10 $\mu$M zolpidem. B. Averages of mIPSCs in control (thin line, 329 events) and in 10 $\mu$M zolpidem (thick line, 313 events). Inset shows both traces normalized to their peak amplitudes. C. Cumulative histograms of amplitude, duration, and interevent interval in control (thin line) and zolpidem (thick line). The mean mIPSC amplitude is $47 \pm 22$ pA in control and $61 \pm 29$ pA in zolpidem, and the mean mIPSC duration is $19.5 \pm 6.5$ msec in control and $32.5 \pm 9.6$ msec in zolpidem. The cumulative distributions of these two parameters in zolpidem are significantly different from the control distributions (KS test; $p < 0.001$). The dotted lines show the calculated distributions of uniformly potentiated control values, which are not significantly different from the distribution in zolpidem (KS test; $p > 0.05$). The cumulative histograms of interevent intervals in control and zolpidem are not significantly different ($p > 0.05$).

**Figure 3.** Concentration-dependent effects of zolpidem on mIPSCs in neocortical pyramidal cells. A, a. Average mIPSCs recorded in a single cell in control conditions and during the application of 0.1, 1, 10, and 100 $\mu$M zolpidem, from left to right. b. The traces are scaled to the control amplitude to show the effect of zolpidem on the duration of the currents. B, Effect of zolpidem on (a) mean mIPSC amplitude and (b) mean mIPSC duration. The values obtained in various concentrations of zolpidem are plotted as ratios over control values. Each point represents the mean $\pm$ SD of the number of cells given in parentheses. The parameters of the fitted sigmoidal curve (see Materials and Methods) are $EC_{50}$, 5.8 $\mu$M; $h$, 0.56; Max., 221%. C, Antagonistic effect of flumazenil (dotted line, 10 $\mu$M) on the effect of zolpidem (thick line, 1 $\mu$M) shown on one cell. Bottom, Summary graph $(n = 5)$ of the effect of zolpidem and flumazenil on the amplitude (open bars) and duration (black bars) of mIPSCs.
as \( I(t) = N_p o(t)^i \), where \( i \) is the current carried by a single channel. Thus the increase of the low mean mIPSC peak amplitude \( I_{\text{peak}} \) by zolpidem (Figs. 2, 3) can be attributable to the increase of these three different terms: \( N_p \), \( P_{o,\text{max}} \), or \( i \). The following experiments were performed to identify which terms are changed by zolpidem.

**Peak-scaled non-stationary variance analysis of mIPSCs**

The elementary current \( i \) can be derived from peak-scaled non-stationary variance analysis of postsynaptic currents (Traynelis et al., 1993; De Koninck and Mody, 1994; Silver et al., 1996). In six pyramidal cells where the frequency was low enough to perform such an analysis (Fig. 4), we found \( i = -1.85 \pm 0.17 \) pA, which leads, taking a reversal potential of 0 mV (Fig. 5B), to a single-channel conductance of 26.4 \( \pm \) 2.4 pS. The mean number of channels open at the peak \( (N_p) \) for this sample is 30.9 \( \pm \) 7.3. When zolpidem (1 or 10 \( \mu \)M) is applied, the elementary current \( i \) remains constant \((-1.88 \pm 0.26 \) pA, 103 \( \pm \) 18% of control value; \( p = 0.8 \)), whereas \( N_p \) is enhanced \((39.1 \pm 7.6, 132 \pm 36% \) of control; \( p = 0.06 \)). Thus we can conclude that the enhancement of mIPSC amplitude by zolpidem is not caused by an increase in the single-channel conductance of postsynaptic GABA\(_A\) receptors. However, because the mIPSCs are highly variable in amplitude (Fig. 1), presumably because the number of channels at different synapses or the amount of GABA released is variable, a scaling procedure was used, and thus it could not be determined by this method whether \( N_p \) or \( P_{o,\text{max}} \) was enhanced. To answer these questions, we used a system in which the number of receptors was constant and we could control the concentration of GABA applied; that is, fast applications of GABA to outside-out patches.

**Fast application of GABA to outside-out patches of layer V pyramidal neurons**

After synaptic release, the neurotransmitter is thought to be present only very briefly at high concentration in the synaptic cleft (Clements et al., 1992). This synaptic concentration transient can be mimicked by short (1 msec) agonist applications to outside-out patches (Colquhoun et al., 1992; Jones and Westbrook, 1995; Galarreta and Hestrin, 1997). To test whether zolpidem can enhance the maximal probability of opening \((P_{o,\text{max}})\) of GABA\(_A\) receptors in conditions similar to those during synaptic release, we first determined at which concentration of GABA the receptors are saturated by a 1 msec application to outside-out patches.

The currents elicited by short pulses (1 msec) of 1 mM GABA are illustrated in Figure 5. The GABA currents are blocked by bicuculline (10 \( \mu \)M; \( n = 3 \)). Their reversal potential is 0 mV \((n = 7)\), corresponding to the chloride equilibrium potential in our recording conditions. On some patches, single-channel openings could be resolved (Fig. 7D) with an elementary current of \(-1.78 \pm 0.19 \) pA \((n = 6)\), leading to a chord conductance of 25.4 \( \pm \) 2.7 pS, which is close to the value found for channels underlying mIPSCs. The decay of these currents can be, like mIPSCs, well fitted by the sum of two exponentials (Table 1).

However, the slowest component is much greater in patches than for mIPSCs, as reported previously in various preparations (Tia et al., 1996; Galarreta and Hestrin, 1997; Mellor and Randall, 1997). The deactivation kinetics of GABA\(_A\) receptor-channels are voltage independent (Fig. 5, Table 1).

NSFA was performed on three patches (Fig. 5C) with saturating applications of GABA (10 mM) (see below). The fits of the variance–mean current gave an elementary current of \(-1.67 \pm 0.02 \) pA (conductance: 23.9 \( \pm \) 0.3 pS). This value is close to the one found for channels underlying mIPSCs and to measured single channel openings. \( P_{o,\text{max}} \) could also be calculated (see Materials and Methods), which gave 0.61 \( \pm \) 0.06 \((n = 3)\). This value is in good agreement with previously reported values for GABA\(_A\) receptors (Jones and Westbrook, 1997).

We constructed a concentration–response curve by taking the response to 1 msec pulses of 1 mM GABA as a reference and changing for one or more other concentrations on the same patch (Fig. 6). We measured an EC\(_{50}\) of 310 \( \mu \)M, a Hill coefficient of 1.74, and a maximal relative response of 1.13, which is close to previously reported values for the same cells (Galarreta and Hestrin, 1997). At a concentration of 10 mM the receptors are saturated, so we took this concentration of GABA to test the effect of zolpidem on \( P_{o,\text{max}} \).

We also examined the changes in the kinetics of the GABA-evoked currents with the concentration of agonist applied. The 10–90% rise time of the currents was reduced as the concentration of GABA increased (Table 1). We did not see any significant
change in the decay kinetics of the GABA-evoked currents for concentrations of GABA between 300 μM and 10 mM. We saw a significant decrease of the mean τ with 100 μM GABA, compared with 1 mM, which elicits only 11 ± 9% of the maximal response, as observed previously (Jones and Westbrook, 1995; Galarreta and Hestrin, 1997).

Effect of zolpidem on outside-out patches of layer V pyramidal cells

We tested the effect of 1 μM zolpidem on currents elicited by various concentrations of GABA for 1 msec on outside-out patches. When the concentration of GABA was not saturating (100 or 300 μM), the amplitude of the current was significantly enhanced by zolpidem, whereas it remained constant when 10 mM GABA was applied (Fig. 7). The rise time of the currents was not affected by zolpidem at all GABA concentrations (Fig. 7B). The effect of zolpidem (1 μM) on the amplitude of the current was approximately the same when 300 μM GABA was applied during 1 msec (141 ± 22% of the control response; n = 4) as its effect on mIPSC amplitude (Fig. 7C). The application of zolpidem did not change the conductance of the channels when single-channel openings were examined (n = 2) (Fig. 7D). The duration of the GABA-evoked currents was also enhanced by zolpidem (133 ± 26, 122 ± 11, and 134 ± 13% of control; p < 0.05, for 0.1, 0.3, and 10 mM GABA, respectively).

These data show that zolpidem does not enhance the maximal open probability of bound GABA receptors in outside-out patches. Therefore, it is unlikely that an increased P_o,max could account for the increase of mIPSC amplitude observed in the presence of zolpidem. The most likely explanation is that N_b, the number of receptors that have bound the neurotransmitter, is enhanced as a result of an increase of the affinity of GABA to its receptor.

Activation of GABA_α receptors at physiological temperature

We recorded mIPSCs in layer V pyramidal cells in a more physiological situation, i.e., at a temperature of 35°C. When compared with room temperature recordings, the frequency of mIPSCs is increased (12.8 ± 6.3 Hz; n = 8), and their kinetics is
accelerated: the mean rise time is $0.63 \pm 0.16$ msec, and the mean duration is $7.4 \pm 1.8$ msec. The mean amplitude of mIPSCs is also increased ($49.3 \pm 4.5$ pA). In five cells, the elementary current, determined by peak-scaled NSFA, was $2.23 \pm 0.13$ pA, which gives a chord conductance of $31.9 \pm 1.8$ pS, significantly different from the value determined at room temperature ($p < 0.05$). On the other hand, the mean number of channels open at the peak of mIPSCs ($N_p$), calculated as the ratio of the mean current to the elementary current, was not significantly different ($p > 0.2$) at room temperature ($20.2 \pm 5.0$) and at $35^\circ$C ($22.1 \pm 2.0$). This suggests that the degree of occupancy of the receptors is the same at both temperatures.

To examine this latter issue further, we determined the sensitivity of GABA$_A$ receptors to fast applications of GABA on outside-out patches. At $35^\circ$C, the current evoked by 10 mM GABA during 1 msec had a rise time of $0.43 \pm 0.22$ msec ($n = 7$) and a duration of $16.8 \pm 8.0$ msec, which was significantly shorter than at room temperature ($p < 0.001$). In two patches, we estimated $P_{o,\text{max}}$ (see Materials and Methods). Taking $I = -2.2$ pA, measured on single-channel openings in three patches (data not shown), which is also the value determined by peak-scaled NSFA applied to mIPSCs at $35^\circ$C, a value of $P_{o,\text{max}}$ equal to 0.8 was found for both patches. Consequently, we can estimate the number of bound receptors during an mPSC (Silver et al., 1996), $N_o = N_p/P_{o,\text{max}}$, $31 \pm 8$ at room temperature and $28 \pm 3$ at $35^\circ$C, which are not significantly different ($p > 0.2$). Assuming that the number of functional postsynaptic receptors is the same at the two temperatures, then the degree of occupancy of GABA$_A$ receptors during synaptic transmission is similar. Moreover, the amplitude of the current evoked by 300 $\mu$M GABA, which is the measured EC$_{50}$ for such applications at room temperature, was
Table 1. Kinetic parameters of GABA-mediated currents

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Rise time (msec)</th>
<th>% (τ1)</th>
<th>τ2 (msec)</th>
<th>τmax or τ2 (msec)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>mIPSCs</td>
<td>0.81 ± 0.16 b</td>
<td>66 ± 12</td>
<td>27 ± 14</td>
<td>130 ± 3.3</td>
<td>18</td>
</tr>
<tr>
<td>10 mm</td>
<td>0.40 ± 0.11 b</td>
<td>48 ± 8</td>
<td>95 ± 55</td>
<td>53 ± 24</td>
<td>10</td>
</tr>
<tr>
<td>1 mM</td>
<td>0.57 ± 0.17</td>
<td>58 ± 9</td>
<td>109 ± 36</td>
<td>47 ± 14</td>
<td>15</td>
</tr>
<tr>
<td>1 mM (+30 mV)</td>
<td>0.60 ± 0.14</td>
<td>52 ± 14</td>
<td>91 ± 19</td>
<td>43 ± 16</td>
<td>5</td>
</tr>
<tr>
<td>300 μM</td>
<td>0.76 ± 0.12 b</td>
<td>64 ± 11</td>
<td>105 ± 22</td>
<td>39 ± 9</td>
<td>6</td>
</tr>
<tr>
<td>100 μM</td>
<td>0.84 ± 0.20 b</td>
<td>n.d.</td>
<td>n.d.</td>
<td>35 ± 7 c</td>
<td>6</td>
</tr>
</tbody>
</table>

n.d., Not determined.

aThe values are different from the text, because they result from the fit of the average mIPSCs.

bSignificantly different from the rise time of 1 ms GABA-elicited currents (p < 0.01).

The responses to 100 μM GABA were too small to be reliably fitted with exponentials. Therefore we used τ2 to quantify their decay kinetics. It is significantly (unpaired t test, p < 0.05) shorter that the decay of 1 ms GABA-elicited currents.

Effect of zolpidem on GABA_A receptors at 35°C

The effect of bath-applied zolpidem (1 μM) was examined on mIPSCs recorded in layer V pyramidal cells at 35°C (Fig. 9A). As seen at room temperature, zolpidem did not change the frequency of mIPSCs (103 ± 18% of control frequency; n = 6) and enhanced the duration of events (160 ± 30% of control; p < 0.005). The two duration distributions were significantly different in the six cells tested (KS test; p < 0.001). The potentiation of the duration was also uniform, as at room temperature: when the duration distributions in control were scaled to the distributions in zolpidem, the resulting distributions were not significantly different (KS test; p > 0.05) from the one in zolpidem. However, the amplitude of mIPSCs was much less enhanced by zolpidem than at room temperature [108 ± 4% of the control amplitude; when cells are taken individually, a significant difference between the two distributions (KS test; p < 0.05) was detected in only one of six cells].

We then examined whether zolpidem could enhance the amplitude of currents evoked by subsaturating GABA applications. As shown on Figure 9C, the amplitude of the current evoked by 300 μM GABA applications on outside-out patches at 35°C was not enhanced by zolpidem (104 ± 8% of the control amplitude; n = 5), whereas its duration was increased (135 ± 22% of control). Therefore, zolpidem does not seem to be able to reveal the degree of occupancy of GABA_A receptors at high temperature.

DISCUSSION

We have shown that at GABAergic synapses, the BZD agonist zolpidem enhances, in a concentration-dependent manner, both the duration and amplitude of mIPSCs recorded at room temperature. The increase in amplitude is attributable to neither an increase in the conductance of the channels, as demonstrated by peak-scaled NSFA, nor the enhancement of the maximal open probability of GABA_A channels, as shown by the effect of zolpidem on currents evoked by fast applications of saturating doses of GABA to outside-out patches. We therefore propose that zolpidem, by enhancing the affinity of the receptors for GABA, increases the number of receptors bound during transmitter release, indicating that GABA_A receptors are not saturated after release of a single quantum.

Effects of zolpidem on GABA_A receptors

Several observations argue for an increase in affinity, and more specifically, of the binding rate of GABA to its receptor in the presence of BZD agonists (MacDonald and Olsen, 1994). These compounds decrease the EC50 for GABA, without changing the
maximal current evoked by GABA (Sigel and Baur, 1988), and increase the opening frequency of GABA_A channels, without changing their mean open time and burst duration (Study and Barker, 1981; Rogers et al., 1994). Moreover, in agreement with Lavoie and Twyman (1996) and Mellor and Randall (1997), we have shown that at room temperature the amplitude of GABA-elicited currents can be enhanced by BZD agonists only when a subsaturating GABA concentration is applied. Moreover, in agreement with these studies, we have shown that the rise rate of GABA-evoked current is not enhanced by BZD agonists when a saturating GABA concentration is tested, consistent with an effect on the binding rate (Lavoie and Twyman, 1996). However, at 35°C, the amplitude of currents evoked by a subsaturating GABA application was not enhanced by zolpidem (Fig. 9C), providing evidence that the binding rate of GABA is little affected at this temperature. A possible explanation of these results is that the activation energy necessary for GABA binding and channel opening, which determines its activation kinetics (Jones et al., 1998), is lowered by zolpidem at 25°C and is unchanged at 35°C.

BZD agonists also increase the duration of mIPSCs in several neuron types (this study; De Koninck and Mody, 1994; Puia et al., 1994; Ponceur et al., 1996; Mellor and Randall, 1997; Nusser et al., 1997), and that of GABA-elicited currents on outside-out patches [this study; Mellor and Randall (1997); but see Lavoie and Twyman (1996)]. Moreover, it has been shown that the duration of the current elicited by a brief agonist pulse is inversely correlated to the agonist unbinding rate (Jones and Westbrook, 1995; Jones et al., 1998). Therefore, the affinity increase of the GABA_A receptor for its endogenous ligand by zolpidem can be attributed to a concomitant increase of the binding rate and decrease of the unbinding rate.

BZDs have been reported to enhance the conductance of GABA_A channels in dentate gyrus granule cells (Eghbali et al., 1997). However, this effect was seen only when a low concentration (< 5 μM) of GABA was applied, and small conductance openings were observed. During synaptic transmission, however, a high concentration (>100 μM) of neurotransmitter is thought to be experienced by the receptors (Clements et al., 1992), so that this effect would not take place for the mIPSCs. We showed, with peak-scaled NSFA, that the conductance of channels underlying the mIPSCs was not changed by zolpidem. Consistent with this observation, the conductance of channels activated by fast GABA application on outside-out patches was also unchanged by zolpidem.

The effect of zolpidem on mIPSC amplitude decreased at the highest concentration tested (100 μM) (Fig. 4). A bell-shaped dose–response curve of BZD agonists has been reported in several studies using whole-cell applications of agonist (Sigel and Baur, 1988) and in single-channel studies (Rogers et al., 1994; Eghbali et al., 1997). The mechanism of such a behavior is unknown but could involve receptor desensitization (Mellor and Randall, 1997).

**Use of fast applications to outside-out patches as a model synapse**

The method of fast applications to outside-out patches offers the best technical approach currently available to mimic the synaptic release of transmitter. The GABA-elicited currents had fast rise times, like those of mIPSCs, and the single-channel conductance, determined directly or by NSFA, is close to that found for mIPSCs. However, receptors in patches may not behave exactly as in the synapse. The deactivation kinetics of currents evoked by GABA applications on outside-out patches is much slower than that of mIPSCs (this study; Tia et al., 1996; Galarreta and Hestrin, 1997; Jones and Westbrook, 1997) and is voltage independent, unlike that of mIPSCs in the same cells (Sailin and Prince, 1996). Several explanations can be proposed: mechanical disturbance of channels during patch excision or loss of intracellular factors and a different state of phosphorylation (Jones and Westbrook, 1997) may change the behavior of channels; receptors in patches may differ from synaptic receptors (Tia et al., 1996); the concentration in the synaptic cleft may be much lower than usually thought (Galarreta and Hestrin, 1997); and the duration of GABA application, which determines partly the deactivation kinetics of GABA_A receptors (Jones and Westbrook, 1995; Mellor and Randall, 1997), may be longer than the actual timecourse of the transmitter in the synaptic cleft. In any case, the discrepancy lies only in the late part of the response; thus the binding and opening rates are probably the same for synaptic channels and for channels in patches.
Implications for GABAergic synaptic transmission

After observing that zolpidem increased the duration but not the amplitude of mIPSCs in dentate gyrus granule cells at 35°C, De Koninck and Mody (1994) concluded that postsynaptic receptors were saturated by GABA during synaptic transmission. However, Frerking et al. (1995) have shown in culture at 25°C that diazepam, another BZD agonist, potentiates mIPSC amplitudes, and they proposed that the mIPSC amplitude is correlated with the peak concentration of transmitter released in the synaptic cleft and hence that GABA<sub>A</sub> receptors are not saturated during synaptic transmission. It has been shown in various structures that the mIPSC amplitude could be enhanced at room temperature by BZD agonists (DeFazio and Hablitz, 1997; Mellor and Randall, 1997; Nusser et al., 1997). In another study (Poncer et al., 1996), no increase in mIPSC amplitude was seen, suggesting GABA<sub>A</sub> receptor saturation in CA3 pyramidal cells. In cerebellar stellate cells, two populations of GABAergic synapses seem to have different degrees of receptor occupancy. The smallest mIPSCs have their amplitude unaffected by the BZD agonist flurazepam, whereas the amplitude of the largest mIPSCs is enhanced (Nusser et al., 1997). With a different approach, Auger and Marty (1997) reached a similar conclusion, showing on the same cells a negative correlation between the peak open probability of channels at single synapses and the number of channels, which can be interpreted as a lower degree of occupancy in larger synapses. In layer V pyramidal cells, we found that the potentiation of mIPSC amplitude is uniform (Fig. 2), suggesting that the degree of receptor occupancy is not maximal and is similar for all of the synapses with miniature activity. An estimate of the concentration reached by GABA in the synaptic cleft can be made by matching the enhancement of the mIPSC amplitude with that of the GABA-elicted currents using 1 μM of zolpidem (Fig. 7C): it gives a concentration of 300 μM, which is the EC<sub>50</sub> found for the GABA dose–response curve (Fig. 6) (Galarreta and Hestrin, 1997). However, it should be noted that the duration of the application to patches (1 msec) is much longer than estimates of the dwell times of neurotransmitter in the cleft (Clements et al., 1992; Holmes 1995; Clements, 1996); thus the peak concentration could be higher to achieve the same degree of occupancy.

For non-NMDA glutamate receptors, receptor occupancy during quantal synaptic transmission is thought to remain the same (Silver et al., 1996) or diminish as temperature is increased (Tong and Jahri, 1994). Thus we might expect GABA<sub>A</sub> receptors not to be saturated at physiological temperature. Consistent with this prediction, we found that the EC<sub>50</sub> for 1 msec applications is higher at 35°C than at room temperature, and that the mean number of channels open at the peak of an mIPSC is not significantly changed between the two temperatures (also see De Koninck and Mody, 1994). However, we did not see any significant change in the mIPSC amplitude when zolpidem was applied at 35°C, as seen in other cell types (De Koninck and Mody, 1994; Soltesz and Mody, 1994; Poisbeau et al., 1997). We have also shown that zolpidem did not enhance the amplitude of currents evoked by subsaturating GABA concentration on outside-out patches. Therefore, no conclusion can be drawn from the effect of zolpidem on mIPSCs regarding receptor occupancy at physiological temperature.

Our study shows that the postsynaptic GABA<sub>A</sub> receptors expressed by layer V pyramidal cells in visual cortex are not saturated by the release of GABA from a single vesicle, but this may not be the case in all GABAergic synapses (Auger and Marty, 1997; Nusser et al., 1997). Moreover, the release of multiple vesicles at a single active zone could increase receptor occupancy and eventually saturate postsynaptic receptors (Silver et al., 1996). In this latter case the variability of the postsynaptic current is very low. Modulation of the release probability of neurotransmitter could thus regulate both the strength and the variability of synaptic transmission.

REFERENCES


