Dentate Gyrus Basket Cell GABA_A Receptors Are Blocked by Zn²⁺ via Changes of Their Desensitization Kinetics: An *In Situ* Patch-Clamp and Single-Cell PCR Study

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Although GABA type A receptors (GABA_ARs) in principal cells have been studied in detail, there is only limited information about GABA_ARs in interneurons. We have used the patch-clamp technique in acute rat hippocampal slices in combination with single-cell PCR to determine kinetic, pharmacological, and structural properties of dentate gyrus basket cell GABA_ARs. Application of 1 mM GABA (100 msec) to nucleated patches via a piezo-driven fast application device resulted in a current with a fast rise and a marked biexponential decay (time constants 2.4 and 61.8 msec). This decay could be attributed to strong receptor desensitization. Dose–response curves for the peak and the slow component yielded EC₅₀ values of 139 and 24 μ M, respectively. Zn²⁺ caused a marked blocking effect on both the peak and the slow component via a noncompetitive mechanism (IC₅₀ values of 8 and 16 μ M). This led to an acceleration of the slow component as well as a prolongation of recovery from

desensitization. Zn²⁺ sensitivity was suggested to depend on the absence of γ -subunits in GABA_ARs. To test this hypothesis we performed single-cell reverse transcription PCR that revealed primarily the presence of α_2^- , β_2^- , β_3^- , γ_1^- , and γ_2^- subunit mRNAs. In addition, flunitrazepam increased the receptor affinity for its agonist, indicating the presence of functional benzodiazepine binding sites, i.e., γ -subunits. Thus, additional factors seem to co-determine the Zn²⁺ sensitivity of native GABA_ARs. The modulatory effects of Zn²⁺ on GABA_AR desensitization suggest direct influences on synaptic integration via changes in inhibition and shunting at GABAergic synapses.

Key words: rat; GABA receptor; kinetics; desensitization; deactivation; pharmacology; benzodiazepines; flunitrazepam; Zn²⁺; patch-clamp *in situ*; nucleated patch; fast application; single-cell PCR; subunits; mRNA

Inhibitory GABAergic synapses of interneurons counterbalance the excitatory discharge pattern of many neuronal circuits in the CNS. The GABAergic interneurons of the hippocampus mediate both feedforward and feedback inhibition as well as disinhibitory inputs to the principal neurons (Freund and Buzsáki, 1996). Although much is known about kinetic (Edwards et al., 1990; Celentano and Wong, 1994; Jones and Westbrook, 1995; Draguhn and Heinemann, 1996), pharmacological (Westbrook and Mayer, 1987; Buhl et al., 1996; Martina et al., 1996), and structural (Laurie et al., 1992; Wisden et al., 1992; Fritschy et al., 1994; Fritschy and Möhler, 1995) properties of GABA_A receptors (GABA_ARs) on hippocampal principal cells, only a few studies have dealt with interneuron GABA_ARs (Fritschy et al., 1994; Gao and Fritschy, 1994; Fritschy and Möhler, 1995).

The kinetic properties of GABA_AR activation have been studied in a number of areas using either application techniques on excised patches or evaluation of IPSCs. The findings were contradictory with respect to the number of exponential functions necessary to describe the current decay (Edwards et al., 1990;

Draguhn and Heinemann, 1996). Developmental regulation and cell type-specific and subunit-specific dissimilarities have been shown to account for the differences in kinetic parameters (Verdoorn et al., 1990; Puia et al., 1994; Draguhn and Heinemann, 1996; Tia et al., 1996). In the present study we have used a fast-application system to nucleated patches to minimize problems in space-clamp or drug exchange rate for the study of the kinetics and Zn²⁺ sensitivity of GABA-mediated currents.

GABA_AR activity is modulated via several substances, such as benzodiazepines, barbiturates, and steroids (for review, see Kaila, 1994; Thompson, 1994). Another modulator, Zn²⁺ (Smart, 1992), acts heterogeneously on GABA_ARs. Although the receptors of prepyriform cortical neurons are almost insensitive to this cation (Smart and Constanti, 1983), hippocampal pyramidal neurons exhibit a pronounced Zn²⁺ block (Westbrook and Mayer, 1987). In recombinant GABA_ARs, Zn²⁺ insensitivity has been correlated with the presence of γ -subunits (Draguhn et al., 1990; Smart et al., 1991) (but see White and Gurley, 1995). The effects of Zn²⁺ on neurotransmitter receptors might be of functional importance, because Zn²⁺ is present in synaptic vesicles, e.g., in mossy fiber boutons (Haug, 1967), and is released during synaptic activation. Thereafter, it may reach local concentrations of up to 300 μ M (Assaf and Chung, 1984) and may modulate inhibitory and excitatory receptors (Westbrook and Mayer, 1987). Furthermore, synaptically released Zn²⁺ from sprouting mossy fibers (Laurberg and Zimmer, 1981) might play a role under pathophysiological conditions (Buhl et al., 1996).

Because information about the subunits constituting the GABA_ARs of interneurons is scarce, we have combined electro-

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physiological techniques with single-cell reverse transcription (RT)-PCR experiments to correlate functional and structural properties of basket cell GABA_ARs. Particularly, we were interested in the expression of β - and γ -subunits, which have been shown to be important for receptor desensitization, benzodiazepine responsiveness, and Zn²⁺ sensitivity in recombinant cells (Pritchett et al., 1989; Draguhn et al., 1990; Verdoorn et al., 1990).

MATERIALS AND METHODS

Brain slice preparation and basket cell identification. Transverse slices (300- μ m-thick) of the hippocampus were cut from the brains of 15- to 20-d-old Wistar rats using a Vibratome (Campden, Loughborough, England). Dentate gyrus basket cells were visualized by infrared differential interference contrast (IR-DIC) videomicroscopy (Stuart et al., 1993) using a Newvicon camera (C2400; Hamamatsu, Hamamatsu City, Japan) and an infrared filter (RG9; Schott, Mainz, Germany) mounted on an upright microscope (Axioskop FS; Zeiss, Oberkochen, Germany). They were identified by their pyramidal morphology, by their location at the border between the granule cell layer and the hilus, and by their high-frequency firing of action potentials generated after sustained membrane depolarization in the current-clamp mode (Koh et al., 1995).

Patch-clamp recording and fast drug application. Patch pipettes were pulled from borosilicate glass tubing (2.0 mm outer diameter, 0.5 mm wall thickness; Hilgenberg, Malsfeld, Germany). When filled with internal solution, they had a resistance of 2.0–2.5 M Ω . Only neurons with resting potentials negative to -60 mV were used. To obtain nucleated patches (Sather et al., 1992), negative pressure (100–200 mbar) was applied during the withdrawal of the patch pipette.

Functional properties of GABA_ARs were investigated using fast application of agonists and modulators (Colquhoun et al., 1992; Jonas, 1995) to nucleated patches. The double-barrel application pipette was made from theta glass tubing (2 mm outer diameter, 0.3 mm wall thickness, 0.12 mm septum; Hilgenberg), and the piezo-electric element used was a PI-245.50 (Physik Instrumente, Waldbronn, Germany) driven by a P-270 high-voltage amplifier. The perfusion rate was 50–70 μ l min⁻¹. The exchange time (20–80%), measured with an open patch pipette during a change between Na⁺-rich and 10%-Na⁺-rich solution, was 150 μ sec. Fast application experiments were started as soon as possible after patch excision (1–2 min after access to the cell interior was obtained). Agonist pulses were applied every 5–8 sec. If modulating substances were applied together with GABA, these were also included in the control solution of the application tool. After completion of the experiment, the patch was blown off and the zero-current potential was measured; it never exceeded 3 mV.

Membrane currents were recorded using an Axopatch 200A amplifier (Axon Instruments, Foster City, CA). Currents were filtered at 5 kHz using the internal four-pole low-pass Bessel filter of the amplifier. Data were digitized and stored on-line using a CED 1401plus interface (CED, Cambridge, England) connected to a personal computer. The sampling frequency was twice the filter frequency. All recordings were made at room temperature (20–24°C). The traces shown represent averages from two to four sweeps (recovery from desensitization) or averages from 15–30 sweeps (all other traces).

Analysis. The decay time constants of the GABA_AR-mediated current were determined by least-squares fit of the decay phase after the peak current. The desensitization time course was evaluated using a fitting interval of 100 msec for 100 msec pulses; the residual current at that time might reflect incomplete desensitization. The fitting interval of the deactivation time course (1 msec pulses), in contrast, was extended to the return of the current to baseline. The value of the interpolated reversal potential (V_{rev}) and chord conductance ratios ($g_{\Delta V1}/g_{\Delta V2}$; g_{+60mV}/g_{-60mV}) were calculated using the values of the fitted I - V curve at $V_{rev} + \Delta V_{1,2}$.

Values of the dose-response curves for the peak and the slow component were obtained as follows. For the peak, the maximal amplitude of the current response was used, whereas the amplitude of the slow component was the sum of the current described by the decay time constant τ_2 and the residual current at the end of the fitting interval. If currents induced by low GABA concentrations could be well fitted by a monoexponential function, the peak amplitude of this current was used for the construction of the dose-response curve of the slow component. The values from different patches were normalized to the value obtained

with 1 mM GABA, plotted semilogarithmically, and fitted by the Hill equation:

$$I = \frac{I_{\max}}{1 + (K/c)^n}$$

where I is the amplitude of the current, $I_{\max}$ is the peak of saturating GABA current, c is the concentration of GABA, K is the half-maximum concentration, and n is the Hill coefficient. The dose-response curves were normalized to $I_{\max}$.

All numerical values denote mean $\pm$ SEM. Statistical significance was assessed by one-way ANOVA at the 0.01 significance level.

Chemicals and solutions. Slices were superfused continuously with a physiological extracellular solution containing (in mM): 125 NaCl, 25 NaHCO₃, 2.5 KCl, 1.25 NaH₂PO₄, 2 CaCl₂, 1 MgCl₂, 25 glucose, bubbled with 95% O₂ and 5% CO₂. The HEPES-buffered Na⁺-rich external solution used for fast application contained (in mM): 135 NaCl, 5.4 KCl, 1.8 CaCl₂, 1 MgCl₂, 5 HEPES, pH adjusted to 7.2 with NaOH. The internal solution used in fast application experiments contained (in mM): 140 KCl, 10 EGTA, 2 MgCl₂, 2 Na₂ATP, 10 HEPES, pH adjusted to 7.3 with KOH. The intracellular solution used in the PCR experiments contained (in mM): 140 KCl, 5 EGTA, 3 MgCl₂, 5 HEPES, pH adjusted to 7.3 with KOH.

All drugs and chemicals were from Sigma (St. Louis, MO) or Merck (Darmstadt, Germany). Stock solutions of 1 M GABA, 300 mM ZnCl₂, and 10 mM (-)bicuculline methiodide were prepared in HEPES-buffered Na⁺-rich external solution; 1 mM flunitrazepam was prepared in DMSO. Serial dilutions provided the final concentrations given below.

Single-cell RT-PCR. Patch pipettes used for the RT-PCR experiments had tip outer diameters of ~2–3 μ m, corresponding to resistances of 0.8–1.4 M Ω when filled with intracellular solution. The glass tubing for the pipettes was heated before use (250°C, 4 hr), and the intracellular solution was autoclaved; the silver wire connected to the patch electrode was chlorided before each experiment. Cytoplasm and nucleus were harvested into the patch pipette under visual control. Cells were used only when the seal remained intact until the very end of the aspiration and were rejected whenever debris remained attached to the outside of the patch pipette. Controls were performed by applying positive pressure to the pipettes, advancing them into the tissue, and using their contents for RT-PCR. The contents of the patch pipettes were expelled into reaction tubes using a valve-controlled pressure system (N₂, 4 bar). Subsequently, reverse transcription was performed (Monyer and Jonas, 1995).

Coamplification of GABA_AR-subunits was performed by nested hot-start PCR using primer sets for the α -, β -, and γ -subunits. The α_6 -subunit was not tested because of its selective localization in cerebellar granule cells. The sequences of the primers for the first round of PCR were $\alpha 5'$, 5'-TGGAC(TC)CC(AT)GA(TC)AC(AC)TTT(TC)TT-3'; $\alpha 3'$, 5'-GC(ATGC)AT(GA)AACCA(GA)TCCATGGC-3'; $\beta 5'$, 5'-CTGGAT-GA(GA)CAAACTGCAC-3'; $\beta 3'$, 5'-AC(AG)AA(CG)AC(GA)AA-(AG)CA(AC)CCCAT-3'; $\gamma 5'$, 5'-T(TG)AA(CT)AGCAA(CT)ATGGT-GGG-3'; $\gamma 3'$, 5'-TTGATCCA(AG)AA(AGT)GA(CT)ACCCAGG-3'.

In certain experiments primer pairs for the specific amplification of the α_1 -subunit were used. For this reaction the above mentioned 3' primer and the following 5' primer were used for the first amplification: α_1 spec., 5'-GGACAGCCCTCCCAAGATGAAC-3'.

The cycling parameters for the first amplification were 94°C for 5 min, 35 cycles (94°C, 30 sec; 53°C, 30 sec; 72°C, 40 sec), and 72°C for 10 min. One microliter of the first-round PCR was reamplified in a second PCR by using a nested 5' primer and the same 3' primer: $\alpha 5'n$, 5'-AA(AG)TTTGG(GAC)AG(CT)TATGC(TCA)TA-3'; $\beta 5'n$, 5'-GAT-GACAT(CT)GAATTTTACTGG-3'; $\gamma 5'n$, 5'-CACTGGAT(AC)AC-(AGC)AC(GTA)CCCAA-3'. For the specific amplification of the α_1 -subunit, the $\alpha 5'$ -primer was used as the nested primer. The second PCR was performed according to the same program. Both PCR reactions contained a 0.4 mM concentration of each primer, 1.5 mM MgCl₂, 0.6 mM dNTPs, and 2.5 U of *Taq* DNA polymerase in the buffer supplied by the manufacturer (Life Technologies, Eggenstein, Germany). As previously described (Geiger et al., 1995), PCR conditions and different primer pairs were tested extensively to guarantee that under conditions used in these experiments GABA_AR-subunits were amplified with a comparable efficiency. The 3' and 5' primer locations span several introns, thus preventing amplification of genomic DNA.

For the identification of parvalbumin (PV)-positive cells, the primers parv.ex3, 5'-CTGCAGACTCCTTCGACCAC-3', and parv.ex4, 5'-

Figure 1. Deactivation and desensitization kinetics. *A*, GABA (1 mM) was applied for 1 msec to a basket cell nucleated patch. The current decay (τ_{total}) could be described by the sum of two exponentials termed τ_1 and τ_2 , as shown in the *bottom panel* of *A*. The open tip response is displayed above the current traces in this and all other figures. *B*, Application of 1 mM GABA for 100 msec to the same patch resulted in a current flow with similar amplitude, rise time, and decay time constants. *C*, The recovery from desensitization was studied with a protocol of two 1 msec pulses of 1 mM GABA with variable interpulse intervals. The extent of recovery from desensitization was obtained from the amplitude ratio of the second pulse divided by the first (I_2/I_1). The current amplitudes were both measured from the baseline directly before the corresponding agonist application (shown in the *inset*). The ratios obtained from eight nucleated patches were plotted semilogarithmically against the interpulse interval and fitted with a biexponential function.

CTTCAACCCCAATCTTGCCG-3' located in exons 3 and 4, respectively, were used. PCR conditions were as follows: 94°C for 5 min, 40 cycles (94°C, 30 sec; 51°C, 30 sec; 72°C, 30 sec), and 72°C for 10 min. For the second amplification a nested 3' primer (parv.ex4n, 5'-GCGGCCAGAAGCGTCTTTG-3') was used.

Analysis of PCR products by Southern blot. After gel electrophoresis, PCR products were denatured by NaOH and transferred to Hybond N⁺ membranes (Amersham, Braunschweig, Germany). The membranes were hybridized with radiolabeled GABA_AR-subunit-specific oligonucleotide probes (α_1 -SB, 5'-TGAGCGGGCTGGCTCCCTTG-3'; α_2 -SB, 5'-AGAGTCAGAAGCATTGTAAG-3'; α_3 -SB, 5'-AGATTTGTTCTTCCCAAGAG-3'; α_4 -SB, 5'-TGACTTCTCAGGGCCTTTGG-3'; α_5 -SB, 5'-AGACTTGGTGGAAACCATTGG-3'; β_1 -SB, 5'-TTGGACACATCTTGTAGTC-3'; β_2 -SB, 5'-ATGACAATGCAGTCACGGGA-3'; β_3 -SB, 5'-CTGGAGACCAGACGGTGCTC-3'; γ_1 -SB, 5'-CGGAGATGTGTGTGAGAGAT-3'; γ_2 -SB, 5'-GTAGTGAAGACAACCTCTGG-3'; γ_3 -SB, 5'-CTGCAGATGTAGTCACGAT-3') overnight at 37°C and were subsequently washed with 0.5× SSC at 55°C. Standard procedures

for probe labeling, hybridization, and posthybridization washing were used (Sambrook et al., 1989).

RESULTS

Deactivation and desensitization kinetics

For the kinetic experiments we used a standard concentration of 1 mM GABA, a value that seems to reflect the concentration in the cleft after vesicle fusion (>500 μ M) (Jones and Westbrook, 1995). GABA was applied to the nucleated patches for 1 or 100 msec at a holding potential of −60 mV (Fig. 1*A,B*, Table 1). The responses to both pulse protocols were characterized by a fast rise (rise time 20–80% of the peak in the range of 295–775 μ sec) (Table 1). The current decay could be best fitted by the sum of two exponential functions, described by their decay time constants, τ_1 and τ_2 , and relative amplitudes, A_1 and A_2 (Table 1).

Table 1. Kinetic and pharmacological properties of the basket cell GABA_ARs

	<i>n</i>	Rise time _{20%/80%} (μsec)	τ ₁ (msec)	τ ₂ (msec)	A ₁ /(A ₁ + A ₂) (%)
1 mM GABA (1 msec pulse)	11	412 ± 32	2.2 ± 0.1	80.3 ± 3.2	75.3 ± 6.7
1 mM GABA (100 msec pulse)	52	559 ± 12	2.4 ± 0.1	61.8 ± 1.7	78.8 ± 6.3
3 mM GABA (100 msec pulse)	7	474 ± 41	2.1 ± 0.1	53.5 ± 1.6	79.4 ± 6.2
300 μM GABA (100 msec pulse)	2	1017 ± 301	2.5 ± 0.1	49.1 ± 4.2	70.0 ± 17.8
100 μM GABA (100 msec pulse)	5	2714 ± 593		64.7 ± 11.4	
30 μM GABA (100 msec pulse)	5	12001 ± 6654		89.9 ± 24.3	
1 mM GABA + 1 μM Flu (100 msec pulse)	6	508 ± 21	2.4 ± 0.1	73.4 ± 2.7	78.9 ± 6.8
100 μM GABA + 1 μM Flu (100 msec pulse)	4	1298 ± 45	4.4 ± 0.1	70.7 ± 6.4	57.2 ± 4.6
		EC ₅₀ (peak)	Hill coefficient (peak)	EC ₅₀ (slow component)	Hill coefficient (slow component)
GABA dose–response curve	10	139 ± 29 μM*	1.0 ± 0.2	24 ± 2 μM*	2.0 ± 0.4
+ 1 μM flunitrazepam	6	57 ± 6 μM*	0.6 ± 0.0	5 ± 2 μM*	1.8 ± 0.6
+ 100 μM Zn ²⁺	8	167 ± 23 μM	0.8 ± 0.1		
		IC ₅₀ (peak)		IC ₅₀ (slow component)	
GABA predesensitization	8	642 ± 105 nM	1.6 ± 0.4	629 ± 96 nM	1.6 ± 0.3
Zn ²⁺ block dose–response curve	8	8 ± 3 μM	0.5 ± 0.1	16 ± 7 μM	0.8 ± 0.3

*Significant differences ($p < 0.01$).

The amplitude fraction of A₁ was obtained by dividing A₁ by (A₁ + A₂) (Table 1). In 11 patches both short and long GABA pulses were applied, and comparison of the above-mentioned parameters gave no significant differences between the two pulse protocols ($p > 0.1$). The peak current amplitude was variable for 1 as well as 100 msec pulses (range, 169–4772 and 198–5177 pA, respectively). The residual current at the end of 100 msec pulses of 1 mM GABA had an amplitude of $12.4 \pm 3.6\%$ of the peak amplitude. In most experiments, no differences between deactivation and desensitization kinetics were seen (current decay did not change its time constant after the end of the GABA pulse) (Fig. 1*B*).

Application of two 1 msec pulses of 1 mM GABA with variable interpulse intervals resulted in paired-pulse depression reflecting the degree of desensitization. The second pulse gained full amplitude after an interpulse interval of ~ 5 sec ($100.5 \pm 3.2\%$) and was half-maximal after ~ 1 sec ($50.4 \pm 2.8\%$; $n = 8$) (Fig. 1*C*).

The GABA_AR-mediated nature of the currents under study was verified by their reversal potential under symmetrical chloride and their sensitivity to the competitive antagonist bicuculline. Application of 1 mM GABA for 100 msec at holding potentials between -80 and 80 mV resulted in an inwardly rectifying dose–response curve (not shown). The reversal potential (V_{rev}) was 4.9 ± 1.2 mV, and the average chord conductance ratio at $V_{rev} + \Delta V_{1,2}$ ($g_{\Delta V1}/g_{\Delta V2}$; g_{+60mV}/g_{-60mV}) was 0.68 ± 0.03 ($n = 8$). Although τ_2 and the amplitude ratio were voltage-independent, τ_1 showed a significant ($p < 0.01$) decrease at more depolarized potentials (ratio τ_1 at 60 mV divided by τ_1 at -60 mV). Coapplication of bicuculline blocked a 1 msec pulse of 1 mM GABA with an IC₅₀ of ~ 300 nM ($n = 2$), whereas 1 mM GABA with bicuculline concentrations as high as 30 μM for 100 msec resulted in the unbinding of the competitive antagonist (cf. Clements, 1996) and a marked GABA-mediated current (not shown).

GABA dose–response curve and predesensitization

Different GABA concentrations (range 10 μM to 10 mM) were applied for 100 msec to the same nucleated patch. Low concen-

trations resulted in current responses with small amplitudes and amplitude fractions, as well as long rise times and decay constants (Fig. 2*A*). With GABA concentrations ≤ 100 μM, only a single decay time constant could be detected. The concentration dependence of the peak and the slow component are shown in Figure 2*B* and Table 1. The decay constant obtained for GABA concentrations ≤ 100 μM is similar to τ_2 of the biexponential decay found with saturating concentrations (Table 1). Thus, the peak amplitude was used for the construction of the dose–response curve of the slow component under low concentration conditions. Peak and slow component displayed different EC₅₀ values and Hill coefficients, likely reflecting different affinities for GABA (Fig. 2*B*, Table 1).

In predesensitization experiments, low GABA concentrations (range, 10 nM to 10 μM) were applied to the nucleated patch before a 100 msec test pulse of 1 mM GABA (Fig. 2*C*). Plotting the current elicited by this test pulse against the preapplied GABA concentration yielded half-maximal predesensitizing concentrations of 642 and 629 nM, respectively, for peak and slow component (Fig. 2*D*, Table 1). These values were not significantly different (one-way ANOVA; $p > 0.5$).

Zn²⁺ block

Coapplication of 30 μM Zn²⁺ with 1 mM GABA (100 msec; $n = 11$) reduced the amplitude of the peak to $27.2 \pm 3.9\%$ and that of the slow component to $34.1 \pm 4.4\%$ of the control values, respectively. The decay time constants τ_1 and τ_2 , in contrast, behaved completely differently in the presence of Zn²⁺: whereas τ_1 was unchanged ($122.9 \pm 9.9\%$ of control), τ_2 was markedly reduced ($33.7 \pm 1.8\%$ of control), resulting in the disappearance of the GABA-mediated current within 30 msec after the beginning of the pulse (Figs. 3*A,C*, 4*A*).

Zn²⁺ in concentrations between 100 nM and 1 mM was coapplied with 1 mM GABA for 100 msec (Fig. 3*A*). To assess the magnitude of block, a 1 mM GABA test pulse was first applied without Zn²⁺, and the following responses were normalized to this one. A dose–response curve for the peak and the slow

Figure 2. GABA dose–response curve. *A*, Different concentrations of GABA (as indicated) were applied for 100 msec to the same nucleated patch. The current responses were superimposed to show their different kinetic behavior. Responses to concentrations ≤ 100 μ M were dominated by a slow rise and decay time constant. *B*, Amplitudes for peak and slow component from 10 patches (I_{GABA}) were normalized to that obtained at 1 mM GABA and were plotted semilogarithmically against the applied GABA concentrations. These data points were used to construct dose–response curves for both components. In both curves, each point represents 1–10 agonist applications. *C*, Before application of 1 mM GABA for 100 msec, different low GABA concentrations (as indicated) were preapplied to the patch via the control barrel of the fast application tool to predesensitize the GABA_A receptors. The current responses were superimposed to make them comparable. All data except the trace with 10 μ M GABA preapplication are from the same patch. *D*, The data from eight nucleated patches were used for the construction of semilogarithmical dose–response plots of the current induced by 1 mM GABA (I_{GABA}) against the preapplied GABA concentration. The plots for peak and slow component are almost identical, i.e., there were no significant differences between the peak and the slow component regarding the GABA predesensitization. Each point represents two to six experiments.

component yielded IC_{50} values of 8 ± 3 and 16 ± 7 μ M, respectively. They were not significantly different ($p > 0.1$) (Fig. 3*B*, Table 1). To test the mechanism of Zn^{2+} block, different GABA concentrations (in the range of 10 μ M to 10 mM) were coapplied with the almost saturating Zn^{2+} concentration of 100 μ M and normalized to the response to 1 mM GABA alone (Fig. 3*C*). The dose–response curve of the peak (Fig. 3*D*) showed an unchanged EC_{50} for GABA (167 ± 23 vs 139 ± 29 μ M under control conditions) (Table 1), whereas the I_{max} was reduced to 19% of the control. These data indicate a noncompetitive blocking mechanism.

To further investigate the possibility of a direct channel block by Zn^{2+} , 1 mM GABA was applied at holding potentials of -60 and 60 mV, either alone or together with 30 μ M Zn^{2+} . The inward rectification of the current–voltage relationship found in control conditions was present also in the presence of Zn^{2+} . The

fraction of the currents blocked by Zn^{2+} was the same at both holding potentials (ratio block -60 mV/ 60 mV = 1.06 ± 0.02 , $n = 5$; mean $\pm$ SEM; not shown). Therefore, the Zn^{2+} block is voltage-insensitive as shown previously by Mayer and Vyklicky (1989) and Draguhn et al. (1990), and these data do not suggest the possibility of a voltage-sensitive channel block.

Subsequently, we tested whether the blocking effect of Zn^{2+} on τ_2 was related to a change of the recovery from desensitization. Therefore, the paired-pulse paradigm from Figure 1*C* was used to estimate the degree of desensitization. GABA at 1 mM ($n = 4$), 1 mM plus 30 μ M Zn^{2+} ($n = 4$), and 30 μ M ($n = 3$) was applied for 20 msec each, with increasing interpulse intervals (Fig. 4*A*). Application of 30 μ M GABA for 1 msec resulted in very small currents; therefore longer pulses were used. Although the recovery from desensitization was complete after an interpulse interval of ~ 5 sec for each of the three experiments ($97.3 \pm 0.6\%$ of the

Figure 3. Zn²⁺ blocks the GABA-induced current via a noncompetitive mechanism. *A*, GABA (1 mM) was applied for 100 msec either alone (*control*) or in combination with different Zn²⁺ concentrations (as indicated). *B*, From eight nucleated patches a semilogarithmic dose–response curve for the Zn²⁺ block was constructed for the peak current and the slow component, respectively. In both curves, each point represents one to eight agonist applications. *C*, Different GABA concentrations (as indicated) were applied for 100 msec in the presence of 100 μM Zn²⁺ to the same nucleated patch to study the mechanism of blockade. GABA (1 mM) was first applied without Zn²⁺ as control. *D*, A semilogarithmic dose–response curve for GABA in the presence of Zn²⁺ was constructed from eight nucleated patches showing the noncompetitive mechanism of block. The peak currents (I_{GABA}) were normalized with respect to the response activated by 1 mM GABA alone. Although the EC_{50} was not changed significantly in comparison to GABA alone, the I_{max} was reduced to 19% of the original value (see *dashed* control dose–response relationship taken from Fig. 2*B*). Note the calibration of the y-axis. Application of 1, 3, and 10 mM GABA yielded significantly smaller responses in combination with 100 μM Zn²⁺ than without (*, one-way ANOVA; $p < 0.01$). Each point represents four to eight experiments.

first pulse amplitude for 1 mM GABA, $95.3 \pm 4.3\%$ for 1 mM GABA plus 30 μM Zn²⁺, and $96.0 \pm 0.6\%$ for 30 μM GABA), the second pulse evoked half-maximal amplitude after different times (Fig. 4*B*). With a 1 sec interpulse interval, 1 mM GABA reached $53.3 \pm 1.6\%$ of the test response (identical to the $50.4 \pm 2.8\%$ found in Fig. 1*C*), 1 mM GABA plus 30 μM Zn²⁺ reached $39.0 \pm 3.5\%$, and 30 μM GABA reached $59.3 \pm 1.2\%$. Thus, coapplication of Zn²⁺ slowed the recovery from desensitization (Fig. 4*B*). This could be attributable to a change of the rate constants in favor of the desensitized states or a disadvantage of the open states. Paired-pulse application of 30 μM GABA, in contrast, showed a faster recovery from desensitization (Fig. 4*B*). The differences for 1 mM GABA plus 30 μM Zn²⁺, and 30 μM GABA, respectively, in comparison to 1 mM GABA were statistically significant for interpulse intervals between 50 and 700 msec

(one-way ANOVA; $p < 0.01$). The biexponential course of the recovery curve suggests the presence of two mechanisms for the recovery from desensitization. (Figs. 1*C*, 4*B*).

The high Zn²⁺ sensitivity of the basket cell GABA_ARs suggests the possibility that they may not contain γ-subunits (Draguhn et al., 1990; Smart et al., 1991). To check the expression of γ-subunits (i.e., functional benzodiazepine binding sites) (Pritchett et al., 1989) we used flunitrazepam as a pharmacological tool. For this purpose, GABA dose–response curves were obtained in the presence of 1 μM flunitrazepam and compared with the curves shown in Figure 2*B*. The only significant effect of the coapplication of flunitrazepam was that even responses to GABA concentrations as low as 100 μM showed two decay time constants (Fig. 5*A*, Table 1). All other kinetic parameters were unchanged when application of 100 μM and 1 mM GABA with and without 1 μM

Figure 4. Zn²⁺ slows the recovery from desensitization of the GABA_ARs. *A*, GABA (1 mM) (■), 1 mM GABA plus 30 μM Zn²⁺ (▲), and 30 μM GABA (●) were applied to the same nucleated patch. Zn²⁺ was applied before and together with the agonist. Although the current responses to 1 mM GABA with and without Zn²⁺ showed almost identical rise times, 30 μM GABA had a much slower onset. All three current responses had different decay kinetics: 1 mM GABA decayed biexponentially, 30 μM GABA decayed monoexponentially, and 1 mM GABA plus 30 μM Zn²⁺ showed a much faster decay of the slow component in comparison to control. The response to 30 μM GABA showed differences between desensitization and deactivation kinetics. The recovery from desensitization was studied with a protocol of two 20 msec pulses with variable interpulse intervals. Current traces with 300, 1000, and 5000 msec interpulse intervals are shown. *B*, The extent of recovery from desensitization was obtained from the amplitude ratio of the second pulse divided by the first (I_2/I_1) (also see Fig. 1C). The ratio obtained from four nucleated patches was plotted semilogarithmically against the interpulse interval and fitted with a biexponential function. The different times needed to obtain half-maximal recovery are indicated by dashed lines for the three conditions. ■, 1 mM GABA; ▲, 1 mM GABA plus 30 μM Zn²⁺; ●, 30 μM GABA.

flunitrazepam were compared (one-way ANOVA; $p > 0.05$). This suggests an increase in the affinity of the GABA_ARs to the agonist that was also reflected in a shift to the left of the dose–response curve for peak and slow component (Fig. 5B, Table 1). The potentiation of GABA in the presence of flunitrazepam was statistically significant for 10 μM and 30 μM GABA (one-way ANOVA; $p < 0.01$), but not for higher GABA concentrations.

Single-cell expression of GABA_AR-subunit mRNA

To determine directly the molecular basis of the previous findings, we used single-cell RT-PCR (Monyer and Jonas, 1995) to detect GABA_AR-subunit mRNAs. Reverse transcription of single-cell mRNA was followed by PCR set-up to coamplify the subunits belonging to one subunit family. For this purpose we designed degenerate primers for the α-, β-, and γ-subfamilies, because the divergence at the nucleotide level in the conserved regions did not permit the design of optimal primers for the concomitant amplification of all subunits. All primers were tested

extensively on total brain cDNA and on plasmids containing different subunit cDNAs to exclude preferential amplification of subunits. Because the multiplex PCR resulted primarily in the amplification of the β-subunit family, the expression profile of the three subfamilies had to be tested on different sets of basket cells. Cells without any PCR product were rejected, and the cells in which PCR products were obtained ($n = 14$ cells for the α-subunits; $n = 12$ for the β-subunits; and $n = 12$ for the γ-subunits) were analyzed by Southern blot hybridization, using probes specific for each GABA_AR-subunit. This resulted predominantly in the expression of the α₂-, β₂-, β₃-, γ₁-, and γ₂-subunit mRNAs (Table 2). All other subunits were expressed less frequently. For the α-subunits, the percentage of cells in which subunit mRNA was detected was 21% for α₁, 86% for α₂, 14% for α₃, 50% for α₄, and 36% for α₅. For the β- and γ-subunits the values were 42% for β₁, 83% for β₂, 100% for β₃, 83% for γ₁, 100% for γ₂, and 58% for γ₃. This high abundance of γ-subunits fitted well

Figure 5. Flunitrazepam shifts the EC_{50} for GABA to lower concentrations. *A*, Flunitrazepam ($1 \mu M$) was coapplied with different GABA concentrations (as indicated) to the same nucleated patch. Even concentrations as low as $100 \mu M$ GABA induced a biexponential response. *B*, The peak amplitudes (I_{GABA}) from six patches were plotted semilogarithmically against the applied GABA concentrations ($+1 \mu M$ Flu). These data points were used to construct the sigmoidal dose–response curve. In a similar way the semilogarithmical dose–response curve of the slow component was constructed. For better comparison the original GABA dose–response relationships without flunitrazepam (*control*; taken from Fig. 2*B*) are shown as *dashed curves*. In both curves each *point* represents one to six experiments. Application of 10 and $30 \mu M$ GABA yielded significantly higher responses in combination with $1 \mu M$ flunitrazepam than without (*, one-way ANOVA; $p < 0.01$).

with the strong modulation of basket cell GABA_ARs by benzodiazepines.

The α_1 -subunit protein has been reported to be strongly expressed in parvalbumin (PV)-positive basket cells (Gao and Fritschy, 1994). We detected this subunit in a subset of the basket cells (21%) tested in this study after amplification with the degenerate primers. To confirm these results we designed a specific primer for the α_1 -subunit, and the PCR analysis of an additional 18 basket cells resulted in the detection of the α_1 -subunit in 38% of the cells. To test the percentage of PV-positive basket cells, PV primers were designed, and single-cell PCR resulted in the expression of this gene in 22% ($n = 2$ of 9 tested) of the cells.

Hence, these data suggest that the α_1 -subunit is expressed in PV-positive basket cells.

DISCUSSION

Kinetic properties of GABA_A receptors

The present study describes the kinetics of GABA_AR-mediated currents in nucleated patches of dentate basket cells and their modulation by Zn^{2+} . In other studies, native GABA_ARs show multiexponential (Edwards et al., 1990; Celentano and Wong, 1994; Maconochie et al., 1994; Jones and Westbrook, 1995; Galarreta and Hestrin, 1997; Mellor and Randall, 1997b) as well as monoexponential (Otis and Mody, 1992; Puia et al., 1994;

Table 2. Subunit composition of the basket cell GABA_ARs

Cell no.	α_1	α_2	α_3	α_4	α_5	β_1	β_2	β_3	γ_1	γ_2	γ_3
1		+									
2				+							
3		+									
4		+									
5		+									
6		+	+	+	+						
7	+	+	+	+							
8	+	+									
9	+	+		+							
10		+									
11		+		+	+						
12		+		+	+						
13		+		+	+						
14					+						
15							+	+			
16						+	+	+			
17						+	+	+			
18						+	+	+			
19							+	+			
20							+	+			
21								+			
22							+	+			
23							+	+			
24						+		+			
25						+	+	+			
26							+	+			
27									+	+	+
28									+	+	
29									+	+	+
30									+	+	+
31									+	+	+
32									+	+	+
33										+	
34									+	+	
35									+	+	
36									+	+	+
37									+	+	+
38										+	

This table shows the results from 38 cells that were amplified with degenerate primers for one of the three GABA_AR subunit families (cells 1–14, 15–26, and 27–38 were amplified with α , β , and γ subunit-specific primers, respectively).

Soltész and Mody, 1994; Draguhn and Heinemann, 1996) decay. In recombinant GABA_ARs, fast application of saturating GABA concentrations always results in a current response with a biexponential decay. The ratio and time course of decay, in contrast, are related to the presence of different GABA_AR-subunits (α_1 vs α_3 , Verdoorn, 1994; Gingrich et al., 1995; β_2 vs γ_2 , Verdoorn et al., 1990). This heterogeneous impression is intensified by the fact that decay kinetics seem to depend on the recording configuration or differences between extrasynaptic versus synaptic receptors (Purkinje cell whole-cell recordings vs fast application to outside-out patches) (Puia et al., 1994). Thus, conditions of improved clamp control and solution exchange might reveal additional information about the kinetic properties.

Desensitization is defined as the current decay in the presence of agonist, whereas the decay after agonist removal is named deactivation. The apparent lack of differences between current responses to 1 or 100 msec GABA pulses in the present study

suggests that the fast application of high agonist concentrations forces the GABA_AR population into a bound and desensitized state (unbinding becomes slower than reopening) (for kinetic model, see Jones and Westbrook, 1995). Thus, the channels seem to desensitize very strongly if GABA is present for a long time, and they hold onto GABA quite tightly after a short pulse.

The time constants of GABA_AR current decay found in basket cells (2.4 and 61.8 msec) are faster than those described in the majority of the previous studies. Only Edwards et al. (1990) (2.2 and 54.4 msec for miniature IPSCs on dentate granule cells) and Galaretta and Hestrin (1997) (5.1 and 62.4 msec for fast application of 1 mM GABA to outside-out patches of neocortical pyramidal neurons) describe similar values. In contrast to these studies, however, we find that the amplitude of the fast component is larger than that of the slow one. Using a paired-pulse protocol, we studied the recovery from desensitization of the two components found with high GABA concentrations. The strong desensitiza-

tion seen as a large $A_1/(A_1 + A_2)$ fraction is also reflected in the long time needed for complete recovery from desensitization (compare Jones and Westbrook, 1995; Galarreta and Hestrin, 1997; Mellor and Randall, 1997b). Because of the slow desensitization kinetics observed previously, desensitization was thought to be of minor importance for IPSCs. The present results, in agreement with recent reports (Celentano and Wong, 1994; Jones and Westbrook, 1995, 1996; Galarreta and Hestrin, 1997), suggest on the other hand that desensitization might play an important role in determining the IPSC shape.

In basket cells, rise, amplitude, decay, and amplitude fractions are markedly concentration-dependent. An amplification of the current as well as an acceleration of current rise and decay with increasing agonist concentrations is also described by other groups (Celentano and Wong, 1994; Maconochie et al., 1994; Gingrich et al., 1995; Jones and Westbrook, 1995) (but see Galarreta and Hestrin, 1997). At low concentrations, ligand-receptor association is slow and desensitization is in concurrence with activation, resulting in slow rise and decay. At high concentrations, association is fast and activation precedes desensitization. This leads to larger responses with additional transient components. The dose–response curves for peak and slow component yield different affinities for the two components. This has also been observed by Celentano and Wong (1994): the fastest desensitization component had the lowest affinity. The activation of the fast component with its low affinity may be attributable to the use of a fast application system in the present study (also see Galarreta and Hestrin, 1997). The EC_{50} for the second component is in agreement with previously reported values for the fast component [native receptors: range, 20.0–48.7 μ M (Jones and Westbrook, 1995; Mellor and Randall, 1997b); recombinant receptors: 7 and 80 μ M (Gingrich et al., 1995)]. Differences in affinities and kinetics therefore may reflect the differences in the speed of agonist perfusion systems and their ability to drive the receptors into a markedly desensitized state.

To further test the possibility of two receptor populations with different affinities, predesensitization experiments were performed (also see Celentano and Wong, 1994). Preapplication of low GABA doses resulted in an equal block of the peak and the slow component (IC_{50} values of 642 and 629 nM, respectively), suggesting the presence of a single receptor population. The half-maximal predesensitizing GABA concentrations are in the range of the GABA concentrations that have been measured in the hippocampal extracellular space (200–800 nM) (Lerma et al., 1986; Tossman et al., 1986) and the cerebrospinal fluid (66–177 nM) (Perry et al., 1982; Schaaf et al., 1985). Hence, it is possible that GABA_ARs *in vivo* can be desensitized partially by tonic GABA, depending on the efficiency of GABA uptake mechanisms at the synapse.

The Zn²⁺ block of native interneuron GABA_A receptors and its functional implications

Zinc ions induce a complex pattern of blockade and potentiation on glutamate and GABA_A receptors. At micromolar concentrations they potentiate AMPA receptor-mediated currents, whereas they block NMDA receptor and GABA_AR responses (Westbrook and Mayer, 1987; Rassendren et al., 1990; but see Hollmann et al., 1993); in millimolar concentrations they block AMPA receptors (Rassendren et al., 1990).

A high-affinity block of GABA_AR-mediated currents by Zn²⁺ was correlated with the absence of γ -subunits (IC_{50} = 560 nM for a $\alpha_1\beta_2$ -subunit combination and >100 μ M for a $\alpha_1\beta_2\gamma_2$ -subunit

combination) (Draguhn et al., 1990). In contrast, White and Gurley (1995) showed IC_{50} values between 1.2 and 9 μ M for γ_2 -containing recombinant receptors. In native receptors, the IC_{50} values show a wide range [5.8–300 μ M (Martina et al., 1996; Hollrigel and Soltesz, 1997)], which was attributed to the different abundance of γ -subunits. These results, together with the IC_{50} values obtained in our study (8 and 16 μ M), made the single-cell RT-PCR study of GABA_AR mRNAs mandatory. The PCR showed the presence of the γ_2 -subunit mRNA in all cells investigated. To confirm the presence of the functional γ -subunit protein in somatic patches, we tested the effect of flunitrazepam. It shifted the GABA dose–response curve to the left and confirms thereby the existence of functional benzodiazepine binding sites pointing to the incorporation of γ -subunits in the GABA_AR molecules (Pritchett et al., 1989). Thus, native basket cell GABA_ARs are Zn²⁺-sensitive and contain γ -subunits.

Zn²⁺ exerts its block via two effects: an acceleration of the slow decay component and a prolongation of the recovery from desensitization. The slow component is correlated with the reopening of the GABA_AR pore (Jones and Westbrook, 1995). A reduction of these reopenings and a simultaneous amplification of the desensitization via changes in the corresponding rate constants would result in the described block pattern. The dramatic effects of Zn²⁺ on the transferred charge (Figs. 3A,C, 4A) (see also Mellor and Randall, 1997a) may have enormous implications on the GABA-mediated IPSCs. If granule cells show just a little activity, Zn²⁺ from their glutamatergic mossy fiber terminals would have only minute or no effects on the postsynaptic basket cell GABA_ARs. High granule cell activity, in contrast, may lead to local Zn²⁺ concentrations of up to 300 μ M (Assaf and Chung, 1984). These zinc ions may spill over to neighboring GABAergic synapses on the basket cell and may disinhibit them. This mechanism could secure the feedback inhibition of the granule cells via their presynaptic basket cells during high activity states.

Structure function relationship of basket cell GABA_ARs

The most prominently expressed GABA_AR-subunit mRNAs in dentate basket cells are α_2 , β_3 , γ_2 . Only one immunocytochemistry study deals with the structural components of putative dentate gyrus basket cell GABA_ARs (Fritschy and Möhler, 1995) showing a prevalence of α_1 -, $\beta_{2,3}$ -, and γ_2 -GABA_AR-subunits for this cell type and other hilar interneurons. In addition to immunohistochemistry, single-cell PCR provides the possibility for describing the structural components of the GABA_ARs of a characterized interneuron population.

Gao and Fritschy (1994) found an expression of the α_1 -subunit in a subset of putative basket cells, namely the PV-positive cells. This contrasts with the dominance of α_2 in hippocampal principal cells (Fritschy and Möhler, 1995). In identified basket cells we found a subpopulation of α_1 -subunit mRNA-containing cells (21% and 38% using the degenerate primer set and α_1 spec, respectively). Because of the lack of detailed data on the abundance of PV-positive basket cells (Ribak et al., 1990; Freund and Buzsáki, 1996), single-cell PCR was performed to assess the percentage of PV mRNA-containing basket cells. With this approach, 22% of the harvested cells contained PV mRNA. Thus, these data indicate the presence of at least two distinct subpopulations of basket cells and are in accordance with previous studies demonstrating the presence of α_1 in PV-positive basket cells. Despite this heterogeneity, the basket cells studied here did not differ with respect to electrophysiological properties.

In addition to providing the main inhibition in the CNS, basket cells and other GABAergic interneurons play a critical role in synchronizing the activity of large ensembles of principal cells. A comprehensive molecular and functional characterization of identified interneurons will ultimately lead to a better understanding of the importance of the large diversity of GABAergic cell types and their significance in the control of network activity.

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