

## Benzodiazepine and $\beta$ -carboline regulation of single GABA<sub>A</sub> receptor channels of mouse spinal neurones in culture

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1. The effects of the benzodiazepine receptor agonist, diazepam (DZ), and the inverse agonist, methyl-6,7-dimethoxyl-4-ethyl- $\beta$ -carboline-3-carboxylate (DMCM), on  $\gamma$ -aminobutyric acid (GABA<sub>A</sub>) receptor single channel currents were characterized. Outside-out patches were obtained from somata of cultured mouse spinal cord neurones and voltage clamped at  $-75$  mV ( $E_{Cl} = 0$  mV).
2. GABA ( $2 \mu\text{M}$ ) alone or with DZ (20–1000 nM) or DMCM (20–100 nM) was applied to patches by pressure ejection from blunt micropipettes. DZ enhanced GABA<sub>A</sub> receptor currents with an inverted U-shaped concentration–response curve. Mean steady-state currents were increased by low concentrations of DZ (20–50 nM). At higher concentrations of DZ, the enhancement was diminished. Mean steady-state currents were decreased by DMCM at all concentrations.
3. GABA<sub>A</sub> receptor channels opened most frequently to a 27 pS main conductance level and less frequently to a 19 pS subconductance level. Neither DZ nor DMCM altered the proportion of time spent at either of the conductance levels. The kinetic properties of the main conductance level were studied.
4. Neither DZ nor DMCM altered the mean GABA<sub>A</sub> receptor channel open or burst durations. Sums of three exponential functions were required to fit best open and burst duration–frequency histograms for GABA alone or with DZ or DMCM. No significant changes in the three time constants or areas of the three exponential functions for open or burst duration histograms were produced by DZ or DMCM.
5. With increasing concentrations of DZ up to 50 nM, GABA evoked an increased frequency of channel openings and bursts. With higher DZ concentrations, the magnitudes of the increase in channel opening and burst frequencies were reduced. At all concentrations of DMCM, GABA evoked a decreased frequency of channel openings and bursts.
6. Closed duration–frequency histograms for GABA alone or with DZ or DMCM were best fitted by sums of at least six exponential functions. The three shortest closed duration time constants were unchanged by DZ or DMCM. The three longest closed duration time constants were altered by DZ and DMCM, consistent with alterations in opening frequency.
7. DZ increased and DMCM decreased steady-state GABA<sub>A</sub> receptor current by increasing or decreasing channel opening frequency without altering mean channel open duration. We propose that DZ and DMCM alter GABA<sub>A</sub> receptor current by acting reciprocally to increase or decrease only, respectively, the apparent agonist association rate at the first of two proposed GABA binding steps without altering channel gating. The basis for the altered apparent association rate is discussed.

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The major inhibitory neurotransmitter of the mammalian CNS is  $\gamma$ -aminobutyric acid (GABA). GABA binds to GABA<sub>A</sub> receptors composed of multiple ( $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$  and  $\rho$ ) polypeptide subunits which combine to form chloride ion-selective channels (Barnard, Darlison & Seeburg, 1987; Schofield *et al.* 1987; Pritchett *et al.* 1989). The GABA<sub>A</sub> receptor channel opens primarily to a main conductance level of 27–30 pS (symmetrical 150 mM chloride ion concentration) and less frequently to conductance levels of 19 and 11 pS (Hamill, Bormann & Sakmann, 1983; Bormann, Hamill & Sakmann, 1987). The GABA<sub>A</sub> receptor channel opens in bursts of openings which increase in duration and complexity as GABA concentration is increased (Macdonald, Rogers & Twyman, 1989*a*). Proposed kinetic models of the main conductance level of the GABA<sub>A</sub> receptor channel have contained multiple open and closed states (Macdonald *et al.* 1989*a*; Weiss & Magleby, 1989; Twyman, Rogers & Macdonald, 1990). To account for the complex bursting properties of the receptor channel, a kinetic model has been proposed which contains two binding sites for GABA, three open states and ten closed states (Twyman *et al.* 1990).

Benzodiazepines, barbiturates and neurosteroids enhance GABA<sub>A</sub> receptor currents and bicuculline,  $\beta$ -carbolines, picrotoxin and penicillin reduce GABA<sub>A</sub> receptor currents by binding to specific binding sites on the GABA<sub>A</sub> receptor channel (Olsen, 1987; Macdonald & Twyman, 1991). A number of compounds act at the benzodiazepine receptor to regulate GABA<sub>A</sub> receptor current. Benzodiazepine receptor agonists such as diazepam (DZ) are anticonvulsant and anxiolytic while benzodiazepine receptor inverse agonists such as methyl-6,7-dimethoxyl-4-ethyl- $\beta$ -carboline-3-carboxylate (DMCM) are convulsant and anxiogenic (Braestrup, Schmiechen, Neef, Nielsen & Petersen, 1982; Braestrup, Nielsen, Honore, Jensen & Petersen, 1983). Diazepam increased GABA<sub>A</sub> receptor currents (Choi, Farb & Fischbach, 1977; Macdonald & Barker, 1978; Skerritt & Macdonald, 1984*b*) by increasing channel opening frequency with little effect on channel open duration (Study & Barker, 1981; Rogers & Macdonald, 1986; Vincini, Mienville & Costa, 1987; Twyman, Rogers & Macdonald, 1989*b*). DMCM decreased GABA<sub>A</sub> receptor currents (Polc, Ropert & Wright 1981; Skovgaard Jensen & Lambert, 1983, 1986; Skerritt & Macdonald, 1984*b*) by decreasing the frequency of channel openings with little effect on the mean open duration of the channel (Vincini *et al.* 1987; Rogers, Twyman & Macdonald, 1989).

The bases for benzodiazepine receptor agonist – or inverse agonist – regulation of GABA<sub>A</sub> receptor current are uncertain. In early studies of benzodiazepine and  $\beta$ -carboline actions, it was proposed that benzodiazepines stabilized a closed form of the GABA<sub>A</sub> receptor with a relatively high opening probability while  $\beta$ -carbolines stabilized a closed form of the GABA<sub>A</sub> receptor with a

relatively low opening probability (Braestrup *et al.* 1982). Several alternative mechanisms are possible. Benzodiazepines might (1) increase the association rate or decrease the dissociation rate for one or both of the GABA binding steps, (2) increase the opening rate to or decrease the closing rate from one or more of the open states or (3) decrease rapid GABA<sub>A</sub> receptor channel desensitization. Inverse agonist  $\beta$ -carbolines might be expected to have actions opposite or reciprocal to those of the agonist benzodiazepines.

To determine which of the above kinetic regulatory mechanisms might be used by benzodiazepine receptor agonists and inverse agonists, the effects of DZ and DMCM on the kinetic properties of GABA<sub>A</sub> receptor single channels evoked from excised outside-out patches obtained from mouse spinal cord neurones in cell culture were determined.

## METHODS

### Cell culture

Neurones were obtained from mechanically dissociated spinal cords dissected from 12- to 14-day-old murine fetuses and were grown in cell culture as previously described (Ransom, Neale, Henkart, Bullock & Nelson, 1977). Timed-pregnant mice were killed by cervical dislocation while under CO<sub>2</sub> narcosis immediately prior to removal of the fetuses. Cultures were allowed to grow for 3–5 weeks prior to being used in the experiments.

### Recording solutions

The bathing solution consisted of (mM): 142 NaCl, 8.1 KCl, 1 CaCl<sub>2</sub>, 6 MgCl<sub>2</sub>, 10 glucose, 10 Na-Hepes, (pH 7.4). The intrapipette solution contained (mM): 153 KCl, 1 MgCl<sub>2</sub>, 10 Na-Hepes, 5 EGTA, 1 NaOH, 2 KOH (pH 7.4). The glycine receptor antagonist strychnine (200 nM; Sigma Chemical Co., St Louis, MO, USA) was added to all solutions to block glycine receptor channel currents. These solutions resulted in a chloride equilibrium potential ( $E_{Cl}$ ) of 0 mV and a potassium equilibrium potential ( $E_K$ ) of –75 mV.

### Micropipettes

Patch clamp recording micropipettes were fabricated from microhaematocrit capillary tubes (Fisher Scientific, Pittsburgh, PA, USA) up to 24 h prior to use. Pressure-ejection micropipettes used for the application of GABA, DZ or DMCM were pulled from 1.2 mm pipettes (World Precision Instruments Inc., New Haven, CT, USA) and had tip diameters of approximately 15–25  $\mu$ m.

### Equipment

Single channel patch clamp recordings were obtained using an L/M EPC-7 amplifier (List Medical Instruments, Darmstadt, Germany). Holding potentials and single channel currents were recorded on a video cassette recording system (VCR; Sony SL-2700, 0–20 kHz) via a digital audio processor (modified Sony PCM-501 ES, 14 bit, 44 kHz). The data were simultaneously recorded on a chart recorder (Gould Inc., Cleveland, OH, USA) using a low-pass (3 dB at 2 kHz) 8-pole

Bessel filter (Frequency Devices, Haverhill, MA, USA). The single channel data were played back from the VCR system and digitized (20 kHz, 12 bit, DT 2801 A/D converter, Data Translation, Marlboro, MA, USA) for computer (DOS-based platform) analyses with a low-pass (3 dB at 2 kHz), 8-pole Bessel filter interposed. Digitized data segments ranged in length from 20 s to 13.3 min.

#### Drug application

GABA (Sigma Chemical Co., St Louis, MO, USA) was mixed with distilled water to a 1 mM concentration, aliquoted and frozen. An aliquot of the GABA stock solution was thawed and mixed with bathing solution to yield a GABA concentration of 2  $\mu$ M. DZ (Hoffman-LaRoche, Nutley, NJ, USA) and DMCM (Ferrosan, Copenhagen, Denmark) were initially combined with the solute dimethyl sulphoxide (DMSO) and subsequently diluted with bathing solution such that the final DMSO concentration was always less than 0.01%. DZ (20, 50, 100, 250 or 1000 nM) or DMCM (20, 50 or 100 nM) was mixed in with the bathing solution except when GABA alone was tested. GABA (2  $\mu$ M) alone or with DZ or DMCM was ejected by air pressure pulses (0.75–1.5 lbf in<sup>-2</sup>) from pipettes which were brought up to within 50  $\mu$ m of the patch pipette tip immediately prior to each application. During interim periods, the pressure-ejection pipettes were removed from the bathing solution. In all experiments, DZ or DMCM were added to the bathing medium prior to the experiments. GABA control recordings were obtained from separate experiments with no drug addition.

#### Outside-out patch recording

Excised outside-out patch recording techniques were as described previously (Hamill, Marty, Neher, Sakmann & Sigworth, 1981; Macdonald *et al.* 1989a). Recordings were obtained at room temperature (20–23 °C). The patches were voltage clamped at -75 mV ( $E_{K}$ ). Recordings of currents evoked by applications of GABA alone, GABA with DZ, or GABA with DMCM, respectively, were combined from several applications to the same patch as well as to different patches. All mean summary data and kinetic analyses were obtained from pooled single channel records.

#### Definition of bursts

Bursts were defined as an opening or a group of openings separated by a relatively long closed period (Colquhoun & Hawkes, 1982). For the purpose of analysis, a critical closed time,  $t_c$ , was chosen so that all openings separated by a closed period less than  $t_c$  belonged within a burst, and bursts were separated by closed periods greater than or equal to  $t_c$ . The method of equal proportions, modified to compensate for missed detections, was used to select a  $t_c$  of 5 ms for these data (Colquhoun & Sakmann, 1985; Macdonald *et al.* 1989a).

#### Single channel data analysis, curve fitting and statistics

Single channel data were analysed by computer using a locally written channel detection program (50% threshold crossing criterion) and locally written analysis programs as previously described (Macdonald *et al.* 1989a; Twyman *et al.* 1990). The GABA<sub>A</sub> receptor channel has been shown to open to as many as four current levels (Bormann *et al.* 1987). In the present study, only openings to the 27 pS main conductance level were analysed. Detected openings less than twice the system rise

time (rise time = 130  $\mu$ s) including the low-pass 8-pole Bessel filter (3 dB cut-off at 2 kHz) were treated as unresolved openings. Detected closings less than the system rise time including the low-pass 8-pole Bessel filter were treated as unresolved closings. Only patches with relatively infrequent multiple openings were used for analysis. When simultaneous channel openings were detected, the openings were considered invalid. The durations of the multiple channel openings were deleted from the record.

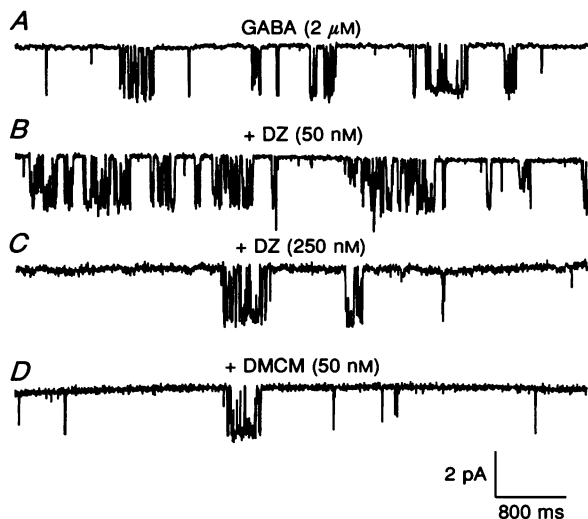
Open and burst durations were placed into frequency histograms using linear binning and closed durations were placed into frequency histograms using logarithmic binning as described previously (Twyman, Green & Macdonald, 1992). Linear frequency histograms were binned to minimize bin promotion errors according to methods previously described (McManus, Blatz & Magleby, 1987; Macdonald *et al.* 1989a). Open durations were binned into 0.5 ms bins with a range of 0.5–50 ms. Inclusion of open durations longer than twice the system rise time only (rise time = 130  $\mu$ s) provided accurate estimation of open durations that reached full amplitude. For logarithmic binning a logarithmic time axis and a square-root ordinate transformation were used (Sigworth & Sine, 1987). Closed durations were binned using a 10 bins per decade resolution with a 200  $\mu$ s minimum duration. Exponential curve fitting was performed using locally written programs described previously (Macdonald *et al.* 1989a; Twyman *et al.* 1990; Stat Library, IMSL Inc., Houston, TX, USA) to find the maximum likelihood estimate of time constants and areas (Colquhoun & Sigworth, 1983). Error ranges for the estimates were calculated using maximum likelihood ranges ( $m = 2$ ) which corresponded to about a 95% confidence interval. The number of significant exponentials was determined by fitting with increasing numbers of exponential functions until (1) the  $\chi^2$  of the estimated fit and the data were within the 95% confidence interval for accepting the null hypothesis (no difference between the estimated fit and data) and/or (2) the maximum likelihood estimate was no longer improved greatly by fitting with additional exponential components (log-likelihood ratio estimates differences of greater than 2 (Akaike, 1974) but preferably greater than 4 (McManus & Magleby, 1989)). Distributions of open, closed or burst durations were fitted over the same ranges for data obtained with GABA alone or with DZ or DMCM.

The mean open and closed durations measured in this study were apparent or observed times. Due to the presence of missed or unresolved openings, the apparent open durations were greater than the true durations (Colquhoun & Sigworth, 1983). Correction of mean open duration for missed openings were obtained by re-estimating the mean open duration from the exponential function fits of the open duration distributions. This type of correction, however, did not correct for the effect of missed closures. A similar correction was made for mean closed duration. Data were presented as means  $\pm$  s.d. unless otherwise indicated.

## RESULTS

### GABA<sub>A</sub> receptor single channel currents

Following excision, outside-out patches were hyperpolarized to -75 mV. GABA (2  $\mu$ M) evoked single and bursting single channel currents (Figs 1A and 2) and



**Figure 1.**

*A*, GABA ( $2\ \mu\text{M}$ ) opened chloride channels resulting in single and bursting inward currents. Time and current calibration bars are applicable to all traces shown. *B*, GABA with DZ ( $50\ \text{nM}$ ) resulted in increased opening frequency. *C*, GABA with DZ ( $250\ \text{nM}$ ) resulted in less of an increase in opening frequency. *D*, GABA with DMCM ( $50\ \text{nM}$ ) resulted in decreased opening frequency. Data were obtained from different excised outside-out patches.

channel openings to two conductance levels of 27 pS (Fig. 2*D*, \*\*) and 19 pS (Fig. 2*D*, \*). The larger main conductance level contributed 97.4% of the single channel current while the subconductance level contributed only 2.6% of the single channel current.

The proportion of time spent in each of the two conductance levels (Figs 1*A* and 2) was not changed by the addition of DZ (Figs 1*B*, *C* and 3) or DMCM (Figs 1*D* and 4), suggesting that any increases or decreases in single channel current produced by DZ or DMCM were due to changes in the temporal characteristics of opening and closing of the channel. Since they contributed the majority of current, only main conductance level openings were investigated in detail.

### GABA<sub>A</sub> receptor single channel currents were enhanced by DZ

Mean GABA<sub>A</sub> receptor single channel currents (Figs 1*A* and 2) were enhanced by DZ (Figs 1*B* and 3) with an inverted U-shaped concentration dependency. Peak enhancement was produced by 50 nM DZ (Table 1*A*). With increases in

DZ concentration above 50 nM, however, mean single channel current was enhanced less (Fig. 1*C*; Table 1*A*).

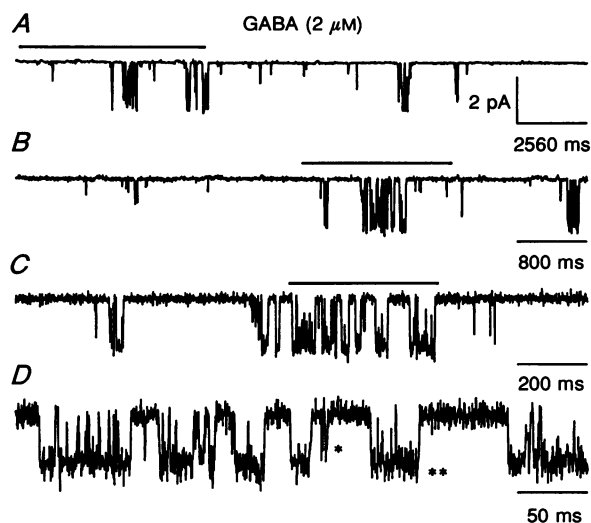
The frequency of GABA<sub>A</sub> receptor single channel currents (Figs 1*A* and 2*A*) was increased by DZ (Figs 1*B* and 3*A*) with an inverted U-shaped concentration dependency. Peak increase in single channel frequency was produced by 50 nM DZ (Table 1*A*). Smaller increases in frequency of single channel currents were produced by concentrations of DZ above 50 nM (Fig. 1*C*; Table 1*A*).

### GABA<sub>A</sub> receptor single channel currents were reduced by DMCM

Mean GABA<sub>A</sub> receptor single channel currents (Figs 1*A* and 2) were reduced by DMCM (Figs 1*D* and 4; Table 1*B*). The frequency of single channel currents (Figs 1*A* and 2*A*) was also decreased by DMCM (Figs 1*D* and 4*A*; Table 1*B*).

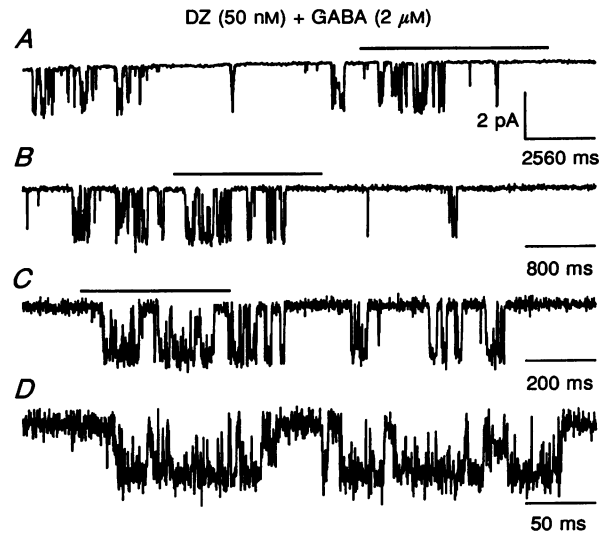
### DZ alteration of GABA<sub>A</sub> receptor single channel open properties

While low concentrations of DZ increased GABA<sub>A</sub> receptor single channel currents (Figs 1*A* and *B*), the temporal



**Figure 2.**

GABA<sub>A</sub> receptor single channel currents evoked by GABA ( $2\ \mu\text{M}$ ) are shown at increasing time resolution. Traces represent samples of channel activity selected to demonstrate typical features of the currents. Portions of channel activity under the horizontal lines are shown at increased time resolution in the trace directly below. Current calibration applies throughout.



**Figure 3.** GABA<sub>A</sub> receptor single channel currents evoked by GABA (2 mM) with DZ (50 nM) are shown at increasing time resolution. See legend of Fig. 2 for details.

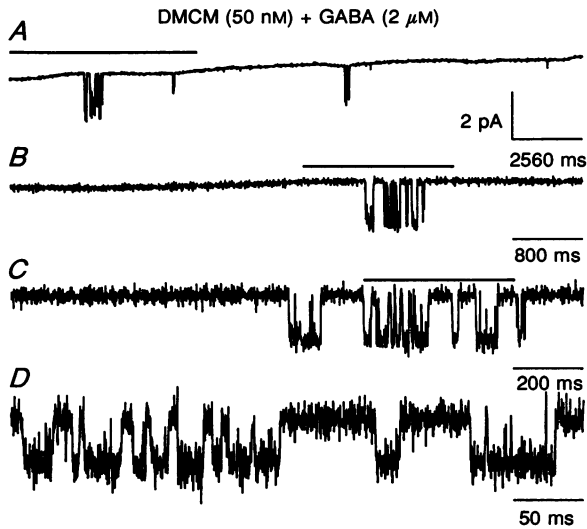
characteristics of individual openings did not appear to be altered (Figs 2 and 3). Mean open duration for single channel openings was 4.1 ms (corrected mean open duration was 3.9 ms) (Table 1A). In the presence of DZ (20–1000 nM, *n* = 5 concentrations), mean open duration for single channel openings averaged  $4.1 \pm 0.38$  ms, range 3.8–4.5 ms (corrected mean open duration  $4.0 \pm 0.36$  ms, range 3.6–4.1 ms; Table 1A).

For GABA, a sum of three exponential functions was required to fit best the open duration frequency distribution (Fig. 5A). The exponential function time constants were 0.67, 2.65 and 7.77 ms and their relative areas were 0.18, 0.49 and 0.33 (Fig. 5B), respectively. There were no consistent differences in the three exponential function time constants or relative areas with any concentration of DZ (Fig. 5B). For DZ (*n* = 5

**Table 1.** Alteration of GABA<sub>A</sub> receptor channel properties by DZ (A) and DMCM (B)

A.						
DZ concentration (nM)	0	20	50	100	250	1000
Mean current (fA)	82	99	216	141	94	91
Opening frequency (s <sup>-1</sup> )	$10.3 \pm 0.6$	$10.5 \pm 0.8$	$21.9 \pm 1.9$	$15.2 \pm 2.0$	$10.3 \pm 1.6$	$6.2 \pm 1.5$
Mean open duration (ms)	$4.1 \pm 0.03$	$4.6 \pm 0.04$	$3.9 \pm 0.03$	$3.8 \pm 0.02$	$3.9 \pm 0.03$	$4.5 \pm 0.02$
Corrected mean						
open duration (ms)	3.9	4.1	3.7	3.6	3.9	4.1
Mean closed duration (ms)	$85.8 \pm 1.5$	$86.8 \pm 2.1$	$30.9 \pm 0.9$	$50.2 \pm 1.2$	$83.4 \pm 2.6$	$100.6 \pm 2.2$
Number of openings	38503	26149	34503	86169	33507	55704
Number of patches	27	30	28	27	30	28
B.						
DMCM concentration (nM)	0	20	50	100		
Mean current (fA)	82	30	29	32		
Opening frequency (s <sup>-1</sup> )	$10.0 \pm 0.6$	$4.8 \pm 0.9$	$3.3 \pm 0.4$	$3.3 \pm 1.3$		
Mean open duration (ms)	$4.1 \pm 0.03$	$4.0 \pm 0.05$	$4.4 \pm 0.07$	$4.6 \pm 0.04$		
Corrected mean						
open duration (ms)	3.9	4.0	4.2	4.5		
Mean closed duration (ms)	$85.8 \pm 1.5$	$263.3 \pm 9.3$	$213.7 \pm 8.7$	$279.1 \pm 10.7$		
Number of openings	38503	13523	8339	21638		
Number of patches	27	30	28	27		

DZ- (A) and DMCM- (B) altered mean GABA<sub>A</sub> receptor single channel open and closed properties. Mean current, opening frequency, mean open duration and percentage of time open were obtained from openings to the 27 pS conductance level. Corrected mean open durations were calculated by taking the sum of the relative areas for each exponential component (*a*) in the open duration histogram multiplied by the time constant (*τ*) of the component (corrected mean open duration =  $a_1\tau_1 + a_2\tau_2 + a_3\tau_3$ ). Data are presented as means  $\pm$  s.e.m.



**Figure 4.** GABA<sub>A</sub> receptor single channel currents evoked by GABA (2 μM) with DMCM (50 nM) are shown at increasing time resolution. See legend of Fig. 2 for details.

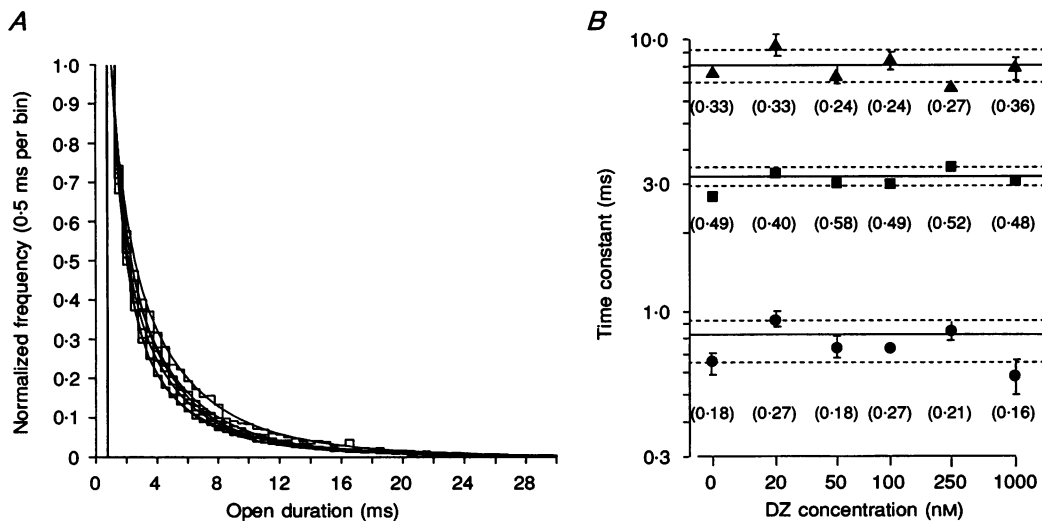
concentrations), time constants averaged  $0.78 \pm 0.13$ ,  $3.22 \pm 0.22$  and  $8.05 \pm 1.08$  ms and relative areas averaged  $0.22 \pm 0.05$ ,  $0.49 \pm 0.07$  and  $0.29 \pm 0.05$  for components 1–3, respectively.

#### DMCM alteration of GABA<sub>A</sub> receptor single channel open properties

While DMCM decreased GABA<sub>A</sub> receptor single channel current (Fig. 1A and D), the temporal characteristics of individual openings varied little following addition of

DMCM (Figs 2 and 4). In the presence of DMCM (20–100 nM), mean open duration for GABA<sub>A</sub> receptor channel openings averaged  $4.2 \pm 0.31$  ms, range 4.0–4.6 ms (corrected mean open duration  $4.2 \pm 0.25$  ms, range 3.9–4.5 ms; Table 1B).

The effects of each concentration of DMCM on the exponential function fits to the GABA open duration frequency histograms were determined (Fig. 6A). There were no consistent differences in the three exponential function time constants or relative areas with any



**Figure 5.**

Open duration–frequency histograms for GABA were not significantly altered by addition of DZ from 20 to 1000 nM. A, frequency histograms were constructed by putting open durations into bins of 0.5 ms over a range from 1 to 50 ms (shown only for 0–30 ms). The distributions were normalized to display relative frequency distributions. Histograms for open durations were fitted with the sum of three exponential functions for GABA (2 μM; uppermost curve) and for GABA (2 μM) with DZ in the following concentrations: 20, 50, 100, 250 and 1000 nM (curves in descending order). The superimposed curves were drawn according to the determined exponential fits. B, open duration time constants and relative areas were obtained by fitting each of the open duration–frequency histograms for GABA and GABA with DZ (20–1000 nM). The histograms were best fitted by sums of three exponential functions whose time constants are presented with 95% confidence intervals. Relative areas are presented in parentheses. Mean time constants (continuous lines)  $\pm$  s.e.m. (dashed lines) obtained with DZ application are shown. See Methods for details of curve fitting.

concentration of DMCM (Fig. 6*B*). For DMCM ( $n=3$  concentrations), time constants averaged  $0.72 \pm 0.16$ ,  $3.02 \pm 0.47$ , and  $7.52 \pm 0.82$  ms and relative areas averaged  $0.24 \pm 0.05$ ,  $0.49 \pm 0.09$  and  $0.27 \pm 0.05$ , respectively.

### DZ alteration of GABA<sub>A</sub> receptor single channel closed times

The closed durations were widely distributed (microseconds to seconds). In the presence of low concentrations of DZ, the mean closed durations between main conductance levels were decreased compared to GABA alone consistent with an increase in opening frequency (Table 1*A*). At DZ concentrations above 50 nM, mean closed duration increased in a concentration-dependent manner consistent with a progressive decrease in opening frequency (Table 1*A*).

The closed duration–frequency histogram for GABA<sub>A</sub> receptor single channel currents was best fitted by six exponential functions (Fig. 7*A*) with time constants ranging from 0.22 to 664 ms (Fig. 7*B*). The relative frequency of occurrence of the briefest to longest openings decreased from 0.32 to 0.04 (Fig. 7*B*). For DZ concentrations up to 50 nM, the closed duration–frequency histograms were shifted to shorter closed durations (Fig. 7*A*). For DZ concentrations above 50 nM, the closed duration–frequency histograms were shifted to longer closed durations (not illustrated). There were few changes in the three shortest time constants with increasing DZ concentration from 20 to 1000 nM (Fig. 7*B*). For GABA, the three briefest time constants were 0.22, 1.46 and 7.25 ms (Fig. 7*B*). With DZ, the three briefest time constants averaged (ms):  $0.25 \pm 0.02$  (range 0.24–0.28),  $1.46 \pm 0.24$  (range 1.07–1.64) and

$7.98 \pm 1.01$  (range 7.27–9.41; Fig. 7*B*). For GABA, the three longest time constants were 46.6, 181 and 664 ms. The three longest time constants varied with DZ concentration (Fig. 7*B*). For DZ concentrations up to 50 nM, these time constants tended to decrease (to 37.6, 129 and 533 ms for 50 nM DZ). For DZ concentrations above 50 nM, these time constants tended to increase (to 80.8, 285 and 1093 ms for 1000 nM DZ).

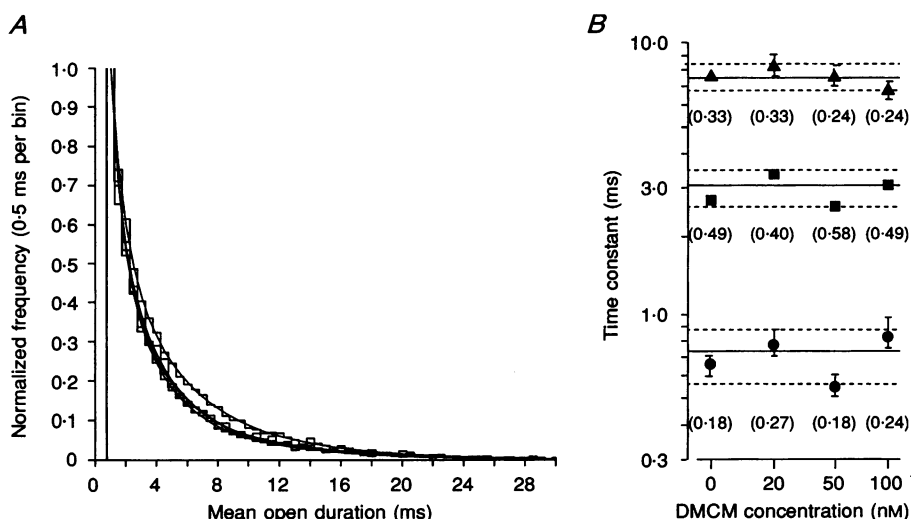
For GABA the relative areas of the six exponential functions ( $a_1$ – $a_6$ ) tended to be largest for the exponential function with the smallest time constant and to decrease for functions with larger time constants (Fig. 7*B*). Addition of DZ (20–1000 nM) did not alter the relative areas of the six exponential functions in any systematic fashion (Fig. 7*B*).

The significance of the longer time constants was unclear since contributions due to multiple channels or desensitization were unknown. However, the tendency of the longer time constants to decrease with increasing DZ concentration up to 50 nM and to increase with DZ concentrations above 50 nM is consistent with the increase in opening frequency produced by low concentrations of DZ and the decrease in opening frequency produced by higher concentrations of DZ.

### DMCM alteration of GABA<sub>A</sub> receptor single channel closed times

In the presence of DMCM, mean closed durations were increased compared to GABA alone, consistent with a decrease in opening frequency (Table 1*B*).

For all DMCM concentrations, the closed duration–frequency histograms were shifted to longer closed

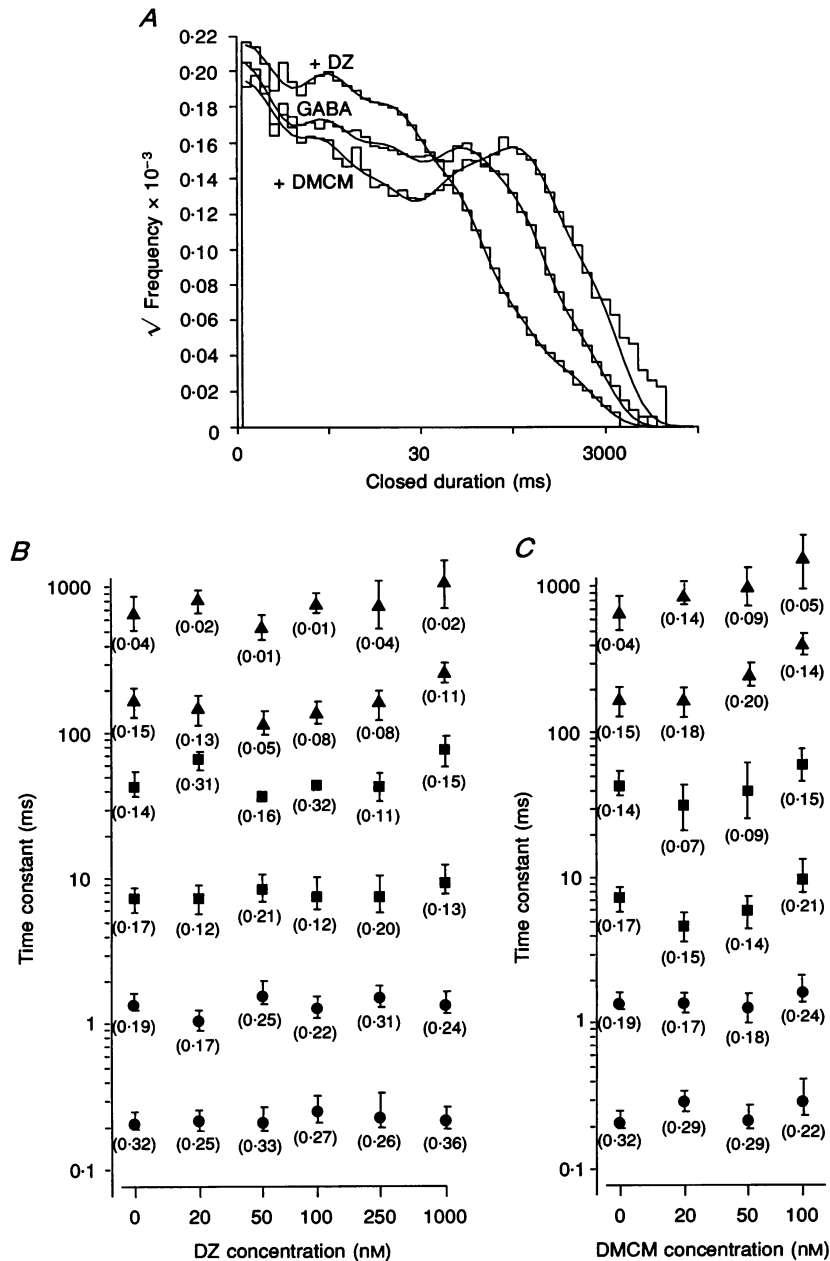


**Figure 6.**

Open duration–frequency histograms for GABA were not significantly altered by addition of DMCM from 20 to 100 nM. *A*, histograms for open durations were plotted for GABA (2 μM; uppermost curve) and for GABA with DMCM in the following concentrations: 20, 50 and 100 nM (curves in descending order). *B*, open duration time constants and relative areas were obtained by fitting each of the open duration–frequency histograms for GABA and GABA with DMCM (20–100 nM). See the legend to Fig. 5 for further details.

durations (Fig. 7A). There were few changes in the three shortest time constants with increasing DMCM concentration (Fig. 7C). For GABA, the three briefest time constants were 0.22, 1.46 and 7.25 ms (Fig. 7C). With

DMCM, the three briefest time constants averaged  $0.29 \pm 0.05$  ms (range 0.23–0.31 ms),  $1.57 \pm 0.27$  ms (range 1.34–1.87 ms) and  $6.87 \pm 4.11$  ms (range 4.56–10.13 ms; Fig. 7C). For GABA alone, the three longest time constants



**Figure 7.**

Closed duration–frequency histograms for GABA ( $2 \mu\text{M}$ ) were altered by addition of DZ (20–1000 nM). A, for clarity of presentation, only data for GABA and GABA with DZ (100 nM) and GABA with DMCM (100 nM) are presented. Histograms were best fitted with sums of six exponential functions and curves were drawn according to the fits (see text). DZ shifted long closed durations to shorter durations while DMCM shifted long closed durations to longer durations. See text for further details and description of effects of other concentrations of DZ and DMCM. B, closed duration time constants and relative areas were obtained by fitting each of the logarithmically binned closed duration–frequency histograms for GABA and GABA with DZ (20–1000 nM). The histograms were best fitted by sums of six exponential functions. See the legend to Fig. 5 for further details. C, closed duration time constants and relative areas were obtained by fitting each of the logarithmically binned closed duration–frequency histograms for GABA and GABA with DMCM (20–100 nM). The histograms were best fitted by sums of six exponential functions. See the legend to Fig. 5 for further details.



were 46.6, 181 and 664 ms (Fig. 7C). The three longest time constants increased with increasing DMCM concentration (to 60.9, 393 and 1670 ms for 100 nM DMCM; Fig. 7C).

For GABA alone, the relative areas of the six exponential functions ( $a_1$ – $a_6$ ) tended to be largest for the briefest function and to decrease for functions with increasing time constants (Fig. 7C). Addition of DMCM (20–100 nM) did not alter the relative areas of the six exponential functions in any systematic fashion (Fig. 7C).

### DZ alteration of GABA<sub>A</sub> receptor single channel burst properties

For GABA, burst frequency was 4.6 s<sup>-1</sup> (Table 2A). Burst frequency was increased by DZ with an inverted U-shaped concentration dependence. Peak increase in burst frequency was produced by 50 nM DZ (Table 2A). At DZ concentrations greater than 50 nM, the increase in burst frequency was reduced.

For GABA, mean burst duration was 10.5 ms (corrected mean burst duration was 11.3 ms; Table 2A). Mean burst duration varied little with DZ (20–1000 nM) and averaged 10.7 ± 1.2 ms (range 9.3–11.7 ms; corrected mean duration 12.2 ± 1.5 ms, range 10.5–14.4 ms).

The mean number of openings per burst evoked was 2.3 for GABA and varied little with DZ, averaging 2.2 ± 0.24 openings per burst (range 1.9–2.5 openings per burst). The

mean intraburst closed time in bursts was 1.0 ms for GABA and varied little with DZ, 1.1 ± 0.07 ms (range 1.0–1.2 ms).

For GABA, a sum of three exponential functions was required to fit best the burst duration frequency distribution (Fig. 8A). The exponential function time constants were 0.60, 4.11 and 21.5 ms and the relative areas were 0.23, 0.31 and 0.46, respectively (Fig. 8B). There were no consistent differences in the three exponential function time constants or relative areas with any concentration of DZ (Fig. 8B). With DZ ( $n=5$  concentrations), time constants averaged 0.71 ± 0.07, 4.27 ± 0.29 and 27.6 ± 6.4 ms and relative areas averaged 0.27 ± 0.05, 0.33 ± 0.08 and 0.40 ± 0.11 for components 1–3, respectively (Fig. 8B).

### DMCM alteration of GABA<sub>A</sub> receptor single channel burst properties

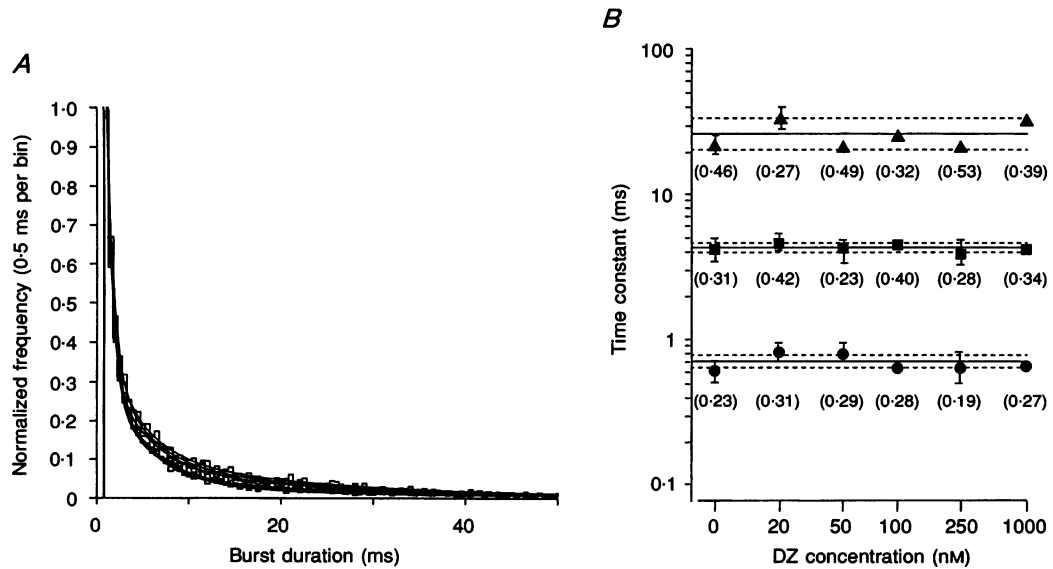
DMCM (20–100 nM) reduced burst frequency (Table 2B). For GABA, mean burst duration was 10.5 ms (corrected mean burst duration was 11.3 ms), was changed little by DMCM (20–100 nM), and averaged 10.3 ± 0.15 ms (range 10.1–10.7 ms; corrected mean burst duration 11.1 ± 0.87 ms, range 10.1–11.7 ms).

The mean number of openings per burst was 2.3 for GABA and was changed little by DMCM, averaging 2.1 ± 0.15 openings per burst (range 1.9–2.2 openings per

Table 2. Alteration of GABA<sub>A</sub> receptor channel burst properties by DZ (A) and DMCM (B)

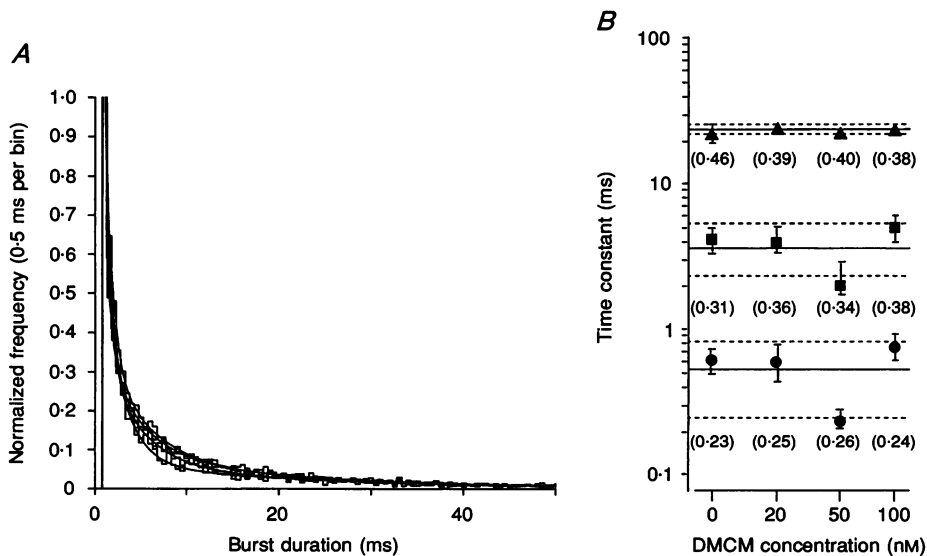
A.						
DZ concentration (nM)	0	20	50	100	250	1000
Burst frequency (s <sup>-1</sup> )	4.6 ± 0.3	5.5 ± 0.4	8.5 ± 0.9	6.2 ± 0.9	3.9 ± 0.6	2.8 ± 0.6
Mean burst duration (ms)	10.5 ± 0.13	9.6 ± 0.16	11.0 ± 0.14	9.3 ± 0.09	11.7 ± 0.16	11.7 ± 0.14
Corrected mean						
burst duration (ms)	11.3	11.7	11.8	10.5	12.7	14.4
Mean number of openings						
per burst	2.3 ± 0.02	1.9 ± 0.02	2.4 ± 0.02	2.1 ± 0.01	2.5 ± 0.02	2.3 ± 0.02
Mean intraburst						
closed time (ms)	1.0 ± 0.01	1.0 ± 0.01	1.1 ± 0.01	1.1 ± 0.01	1.2 ± 0.01	1.1 ± 0.01
Number of bursts	17040	13665	14254	40683	13196	24289
B.						
DMCM concentration (nM)	0	20	50	100		
Burst frequency (s <sup>-1</sup> )	4.6 ± 0.3	2.0 ± 0.3	1.6 ± 0.2	2.0 ± 0.7		
Mean burst duration (ms)	10.5 ± 0.13	10.2 ± 0.26	10.7 ± 0.32	10.1 ± 0.16		
Corrected mean						
burst duration (ms)	11.3	11.5	10.1	11.7		
Mean number of openings						
per burst	2.3 ± 0.02	2.2 ± 0.03	2.1 ± 0.04	1.9 ± 0.02		
Mean intraburst						
closed time (ms)	1.0 ± 0.01	1.1 ± 0.02	1.0 ± 0.03	1.3 ± 0.02		
Number of bursts	17040	6141	4010	11187		

DZ (A) and DMCM (B) altered mean GABA<sub>A</sub> receptor single channel burst properties. Burst frequency, mean burst duration, mean intraburst closed duration, mean events per burst, and the total number of bursts were obtained from detected bursts and the openings and closings within bursts. Bursts were defined by groups of openings separated by closures greater than 5.0 ms. Data are presented as means ± s.e.m.



**Figure 8.**

Burst duration–frequency histograms for GABA were not significantly altered by addition of DZ from 20 to 1000 nM. *A*, burst duration–frequency histograms for GABA (2 μM; uppermost curve) and GABA with DZ in the following concentrations: 20, 50, 100, 250 and 1000 nM (curves in descending order) were placed into frequency histograms with bins of 0.5 ms in the range from 1 to 100 ms (shown only for 0–50 ms). Individual histograms were normalized to display relative frequency histograms. Burst duration–frequency histograms were fitted with the sum of three exponential functions. The superimposed curves were drawn according to the determined burst duration exponential fits. *B*, burst duration time constants and relative areas were obtained by fitting each of the burst duration–frequency histograms for GABA and GABA with DZ (20–1000 nM). The histograms were best fitted by sums of three exponential functions. See the legend to Fig. 5 for further details.



**Figure 9.**

Burst duration–frequency histograms for GABA were not significantly altered by addition of DMCM from 20 to 100 nM. *A*, histograms for burst durations were fitted with the sum of three exponential functions for GABA (2 μM; uppermost curve) and GABA with DMCM in the following concentrations: 20, 50 and 100 nM (curves in descending order). *B*, burst duration time constants and relative areas were obtained by fitting each of the burst duration frequency histograms for GABA and GABA with DMCM from 20 to 100 nM. The histograms were best fitted by sums of three exponential functions. See the legend to Fig. 5 for further details.

burst). The mean intraburst closed time in bursts was 1.0 ms for GABA and was changed little by DMCM, averaging  $1.1 \pm 0.10$  ms (range 1.0–1.2 ms).

For each concentration of DMCM, the burst duration–frequency histograms for GABA were best fitted by the sum of three exponential functions (Fig. 9A). There were no consistent differences in the three exponential function time constants or relative areas with any concentration of DMCM (Fig. 9B). With DMCM ( $n = 3$  concentrations), time constants averaged  $0.53 \pm 0.28$ ,  $3.8 \pm 1.4$  and  $25.0 \pm 1.4$  ms and relative areas averaged  $0.25 \pm 0.01$ ,  $0.36 \pm 0.02$  and  $0.39 \pm 0.01$ , respectively (Fig. 9B).

## DISCUSSION

### DZ and DMCM did not alter GABA<sub>A</sub> receptor single channel conductance

As previously reported (Macdonald *et al.* 1989a), the GABA receptor channel opened to a main conductance level of 27 pS and a subconductance level of 19 pS. The main and subconductance level amplitudes and their relative frequencies of occurrence were unchanged by DZ or DMCM. Since conductance levels were unaltered, it was likely that DZ and DMCM altered either binding or gating properties of the GABA<sub>A</sub> receptor channel.

### DZ and DMCM did not alter GABA<sub>A</sub> receptor single channel open or burst properties

The mean open and burst durations of GABA<sub>A</sub> receptor single channels were unaltered by increasing concentrations of DZ or DMCM. Sums of three exponential functions were required to fit best the open and burst duration–frequency histograms for GABA receptor channel single channel currents, consistent with previous reports that the GABA<sub>A</sub> receptor channel opens into at least three open and burst states with mean dwell times equal to their three exponential time constants (Macdonald *et al.* 1989a, b; Weiss & Magleby, 1989; Twyman *et al.* 1990). Neither DZ nor DMCM produced substantial alterations in the exponential time constants or the relative area of the exponential functions at any concentration used. These results suggest that neither DZ nor DMCM altered (1) the probability of closing of the receptor channel from each of the three open states, (2) the relative frequency of opening of the receptor channel into the three different open states, (3) the probability of opening from each of the intraburst closed states, (4) the probability of intraburst closing from each of the three open states or (5) the relative frequency of occurrence of the three burst states of the GABA<sub>A</sub> receptor channel.

The opening and burst frequencies of GABA<sub>A</sub> receptor channels, however, were increased by low concentrations of DZ (< 50 nM) and were decreased by DMCM at all concentrations up to 100 nM. For DZ the concentration-dependent effect on opening frequency had an inverted

U-shape. With high concentrations of DZ (> 50 nM), the increase in opening and burst frequencies declined in a concentration-dependent manner. The effects of low concentrations of DZ to enhance and of DMCM to reduce GABA<sub>A</sub> receptor current were due entirely to enhancement and reduction of opening and burst frequency, respectively.

### GABA<sub>A</sub> receptor single channel closed properties

A sum of six exponential functions was required to fit best each of the closed duration–frequency histograms for GABA<sub>A</sub> receptor single channel currents. The three longest closed time constants were altered by DZ and DMCM while the three briefest time constants were unaffected by DZ and DMCM. In general, the three longest time constants decreased with increasing DZ concentration up to 50 nM and increased with increasing concentrations above 50 nM. The three longest time constants increased with increasing DMCM concentrations. The changes in long closed time constants were consistent with the effects of DZ and DMCM on opening frequency and suggested that these closed states occur prior to complete occupation of all GABA binding sites. We have reported previously that the two shortest closed duration states were GABA concentration-independent and occurred primarily within bursts (Macdonald *et al.* 1989a; Twyman *et al.* 1990). The finding that the shortest closed time constants and their relative frequency of occurrence were unaltered by DZ and DMCM suggests that DZ and DMCM do not modify opening or closing rates within bursts.

### Comparison with other studies

Using the noise-analysis technique, DZ applied at concentrations from 1 to 17 nM slightly increased GABA<sub>A</sub> receptor single channel open lifetime, but the effect was smaller than necessary to account for the total increase in current (Study & Barker, 1981). It was thus concluded that the main effect of DZ was to increase the frequency of channel opening. GABA application resulted in a single open time constant of 18.3 ms. In the presence of DZ, GABA application also resulted in a single open time constant with durations of 20.8, 25.5 and 29.8 ms as concentration was increased. In these experiments the maximal frequency resolution was 100–200 Hz, and thus, these ‘open durations’ were probably long burst durations rather than open durations. Consistent with these findings, we report that the major effect of low DZ concentrations was to increase opening frequency with relatively little effect on open or burst durations.

The effects of the benzodiazepine, flunitrazepam and DMCM on GABA<sub>A</sub> receptor single channel currents have also been reported (Vincini *et al.* 1987). Flunitrazepam (1 μM) increased GABA-evoked (1 μM GABA) chloride current by increasing opening frequency and DMCM (5 μM)

decreased chloride current by decreasing opening frequency with no effect on open or burst duration. However, only two open duration time constants of 0.4 and 2.5 ms, two closed duration time constants of 0.8 and 18 ms, and two burst duration time constants of 0.4 and 8.0 ms were reported. This is in contrast to our findings of three open duration, six closed duration and three burst duration time constants. It is possible that the numbers of openings and closings in their experiment were insufficient to resolve additional time constants.

### Effect of high concentrations of benzodiazepines

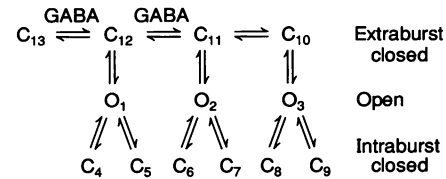
The effect of increasing concentrations of DZ has previously been shown, using current clamp recordings, to result in an inverted U-shaped function (Macdonald & Barker, 1978; Skerritt & Macdonald, 1984*a*). The present study of DZ actions on GABA<sub>A</sub> receptor single channels indicates that the changes in current were due to alterations in the frequency of bursts of channel openings but not due to alterations in the frequency of channel openings within bursts. The basis for the inverted U-shaped DZ concentration–response curve was uncertain. Since DZ increased the frequency of bursts of channel openings, it may also have increased the rate of GABA<sub>A</sub> receptor channel desensitization. It has been shown that DZ increased the rate at which GABA<sub>A</sub> receptor current decreased. Thus, it is possible that the net result of DZ application is dependent on the relative magnitude of enhancement and desensitization. At low DZ concentrations, enhancement is dominant and desensitization is minimal while at higher DZ concentrations desensitization becomes more prominent. However, the experiments performed in the present study were not designed to differentiate between desensitization and decreased opening frequency.

### Site of benzodiazepine regulation of GABA<sub>A</sub> receptor channel gating

Enhancement or reduction of GABA<sub>A</sub> receptor channel current by DZ or DMCM could be produced by drug action at several different sites. Enhancement (reduction) of current could be produced by an increase (decrease) in channel conductance, mean open duration or opening frequency. Neither DZ nor DMCM altered channel conductance. DZ and DMCM altered GABA<sub>A</sub> receptor single channel current by altering opening frequency without altering opening rates, closing rates or burst mechanisms.

A kinetic gating scheme for the GABA<sub>A</sub> receptor channel has been proposed (Scheme 1; Twyman *et al.* 1990). The open and closed states of Scheme 1 are numbered to simplify discussion. Rate constants corresponding to microscopic transition rates between connected states *i* and *j* will be designated  $k_{ij}$ .

The open states correspond to the bound states 1, 2 and 3. Closed extraburst states correspond to the unbound state 13, singly bound state 12 and doubly bound states 10 and 11. Intraburst closed states correspond to singly bound states 4



Scheme 1.

and 5, and doubly bound states 6, 7, 8 and 9. Openings of the open states 1, 2 and 3 had mean open durations of about 0.5, 2.4 and 8.0 ms, respectively. These were GABA concentration independent. However, increasing GABA concentration (0.5–5  $\mu\text{M}$ ) increased both opening frequency and mean open durations (Macdonald *et al.* 1989*a*; Twyman *et al.* 1990). The basis for the increase in mean open durations with increasing GABA concentration was due to an increase in the first two transition rate constants ( $k_{13,12}$  and  $k_{12,11}$ ) due to increased GABA concentration, and therefore, an increase in the relative occurrence of transitions to open states 2 and 3 which have longer mean open durations than open state 1.

In contrast, DZ increased open frequency without altering mean open duration or the relative frequency of occurrence of openings of the three open states. The increase in opening frequency with DZ was therefore produced by a different mechanism. DZ increased the probability of GABA<sub>A</sub> receptor channel opening without altering the kinetics of channel closing or the relative proportion of time spent in any one of the open states once GABA is bound. DMCM decreased the probability of GABA<sub>A</sub> receptor channel opening without altering the kinetics of channel closing or the relative proportion of time spent in any one of the open states after GABA was bound.

Since burst frequency, but not intraburst opening frequency, was altered, it is unlikely that receptor channel opening rates were altered by DZ and DMCM. Therefore, these data were not consistent with the proposal of Braestrup *et al.* (1982) that benzodiazepines stabilize a closed form of the GABA<sub>A</sub> receptor with a high opening probability while  $\beta$ -carbolines stabilize a closed form of the GABA<sub>A</sub> receptor with a low opening probability. Instead, DZ probably increases and DMCM probably decreases the rate of entry into the singly bound closed state 12 without altering any subsequent rate constants. This effect could be produced either by directly altering the rate of binding of GABA or by altering an available pool of GABA<sub>A</sub> receptor channels. If binding rate was altered, it could only be an alteration of the first binding step since a similar alteration of the second binding step would result in an alteration of

mean open and burst durations. If the pool of receptors is altered due to desensitization or other mechanisms, DZ would have to increase and DMCM decrease the number of GABA<sub>A</sub> receptor channels available for binding by GABA. Of the two mechanisms, the former seems more likely since binding studies have not demonstrated an increase in the number of GABA<sub>A</sub> receptor binding sites. However, if a factor which places the GABA<sub>A</sub> receptor in an unavailable pool of receptors is lost in the binding assay, no increase in binding sites by DZ would be expected. If the factor was required for DMCM action, then no decrease in binding sites would be produced by DMCM.

### Comparison of benzodiazepine with barbiturate and neurosteroid regulation of GABA<sub>A</sub> receptor channel gating

The barbiturates phenobarbitone and pentobarbitone increased GABA<sub>A</sub> receptor current by a mechanism distinct from that of DZ (Macdonald *et al.* 1989*b*; Twyman, Rogers & Macdonald, 1989*a, b*). The barbiturates did not increase GABA<sub>A</sub> receptor single channel current by altering opening frequency. Instead barbiturates increased single channel current by increasing mean open duration. The barbiturates produced a shift in the proportion of time spent in the three open states from the brief open states 1 and 2 to the longest open state 3. Barbiturates altered GABA<sub>A</sub> receptor gating and benzodiazepines altered a GABA binding step, and thus, had distinct sites of action on the GABA<sub>A</sub> receptor channel. Recently, several neurosteroids have been demonstrated to enhance GABA<sub>A</sub> receptor current (Majewska, Harrison, Schwartz, Barker & Paul, 1986; Barker, Harrison, Lange & Owen, 1987). We demonstrated that the neurosteroids pregnanolone and androsterone enhanced GABA<sub>A</sub> receptor single channel current primarily by producing a shift in the proportion of time spent in the three open states from the brief open states 1 and 2 to the longest open state 3 and thereby increasing mean open duration. In addition, they also increased opening frequency (Twyman & Macdonald, 1992). Thus, these neurosteroids appeared to share mechanisms of enhancement of both barbiturates and benzodiazepines.

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