Frequency-dependent actions of benzodiazepines on $GABA_A$ receptors in cultured murine cerebellar granule cells

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1. Miniature IPSCs recorded from cultured murine cerebellar granule cells increased in half-width and amplitude following application of the benzodiazepine (BDZ) Flunitrazepam (Flu, 1 μM). The increase in the half-width was much greater than that in the amplitude.

2. Five-millisecond applications of 1 mM GABA to nucleated outside-out patches elicited rapidly rising biexponentially decaying responses that resembled IPSCs. Flu had no effect on the amplitude of such responses, but consistently slowed their deactivation by ~50%. This effect was reversed by Flu washout or application of the BDZ antagonist Ro15-1788. The partial inverse agonist Ro15-4513 speeded deactivation and depressed peak current amplitude by 23 ± 12%.

3. The EC_{50} for GABA was between 45 and 50 μM. At submaximally effective agonist concentrations, Flu increased response amplitude and slowed response deactivation. Both effects were present in all cells taken from young cultures (4–7 days *in vitro*) but the latter was absent in 55% of the neurones obtained from older cultures (14–27 days *in vitro*).

4. With 120 ms applications of 20 μM GABA, responses activated monoexponentially (time constant, 39·8 ± 2·8 ms) and deactivated biexponentially (time constants, 40·4 ± 2·1 and 251 ± 15 ms). Application of Flu slowed both activation and deactivation. The latter effect arose from an increased contribution of the slower component of decay.

5. Desensitization of responses to 1 mM GABA was biexponential, with time constants of 47 ± 11 and 479 ± 49 ms. Flu speeded desensitization by decreasing both fast and slow time constants. $GABA_A$ receptor desensitization consistently slowed subsequent deactivation. No significant relationship between the level of desensitization and the amount of slowing of deactivation produced by Flu was found.

6. Responses to paired 5 ms applications of 1 mM GABA indicated that the slowing of deactivation and the speeding of desensitization produced by Flu combine to generate a marked frequency dependence in the actions of this BDZ. Thus when compared with control responses, GABA-induced charge transfer was only enhanced by Flu during the first of two successive agonist applications.

The kinetic properties of synaptic responses are fundamental in defining their physiological roles (Mody, De Koninck, Otis & Soltesz, 1994). The time course of GABAergic IPSCs has been investigated at a wide variety of synapses. The kinetic properties reported vary somewhat between individual synaptic pathways, species and developmental stages (e.g. Vicini, Alho, Costa, Mienville, Santi & Vaccarino, 1986; Otis & Mody, 1992; Puia, Costa & Vicini, 1994; Frerking, Borges & Wilson, 1995; Tia, Wang, Kotchabhakdi & Vicini, 1996*a*). These differences probably reflect a variety of factors, including the magnitude and duration of the synaptic GABA transient produced following release and the precise subunit make-up of the $GABA_A$ receptors present postsynaptically (Puia *et al.* 1994; Frerking *et al.* 1995; Tia *et al.* 1996*a*; Tia, Wang, Kotchabhakdi & Vicini, 1996*b*). A 'typical' GABAergic IPSC, recorded at room temperature, exhibits an average 20–80% rise time of between about 0·3 and 1 ms. The decay phase of IPSCs mediated by the $GABA_A$ receptor has been reported to proceed either mono- or biexponentially over ~40–300 ms (Mody *et al.* 1994).

Comparison of IPSC waveforms with the kinetics of responses produced by brief applications of exogenous GABA to membrane patches allows inferences to be made regarding the temporal profile of the free GABA concentration in the

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synaptic cleft during neural transmission. Such experiments support theoretical calculations that suggest that the release of the contents of a single vesicle into the synaptic cleft produces an almost instantaneous rise to receptor-saturating levels of GABA (i.e. ~1 mM; DeKoninck & Mody, 1994; Maconochie, Zempel & Steinbach, 1994; Puia *et al.* 1994; Jones & Westbrook, 1995*a*, 1996). The decline from this peak concentration to baseline levels is rapid, reaching completion within about 5 ms (Clements, 1996). A consequence of this is that postsynaptic $GABA_A$ receptors never reach a ligand-bound equilibrium during normal synaptic activity. Thus the properties of GABAergic IPSCs should be considered as arising predominantly from non-equilibrium properties of the $GABA_A$ receptor.

Pharmacological manipulation of $GABA_A$ receptor function has many clinical uses. These include induction of anaesthesia and sleep, treatment of seizure conditions and the alteration of mood. Of the many compounds active at $GABA_A$ receptors, the benzodiazepines (BDZs), such as diazepam, flurazepam and nitrazepam, have been in widespread therapeutic use for over 30 years (Bellantuono, Reggi, Tognoni & Garattini, 1980; Hobbs, Rall & Verdoorn, 1996). *In vivo*, this class of compound commonly produces a range of sedative–hypnotic and/or anxiolytic actions.

Mechanistically, BDZs bind to a specific allosteric site on the $GABA_A$ receptor. Agonist occupancy of this site enhances GABA binding (Costa & Guidotti, 1979) and leads to a leftward shift in the GABA dose–response relationship observed electrophysiologically. This classically produces potentiation of GABA responses at submaximally effective concentrations of GABA (Choi, Farb & Fischbach, 1977; Study & Barker, 1981). In contrast, the amplitude of responses generated by receptor-saturating concentrations of GABA (e.g. 1 mM) are reported to be little altered by co-application of BDZs (Choi *et al.* 1977; Macdonald & Barker, 1978; Farrant, Gibbs & Farb, 1990; however, see Lavoie & Twyman, 1996). These data, along with extensive steady-state single-channel analysis of BDZ actions (Vicini, Mienville & Costa, 1987; Macdonald & Twyman, 1992; Rogers, Twyman & Macdonald, 1994), have produced the widely accepted hypothesis that BDZs act by increasing the microscopic binding rate of the first GABA molecule to the $GABA_A$ receptor complex (Lavoie & Twyman, 1996). The majority of experiments leading to this hypothesis have been performed under near steady-state conditions. These experimental regimes may bear little relevance to the non-equilibrium transitions that underpin GABAergic synaptic responses. Also, such approaches can overlook changes in non-equilibrium properties of $GABA_A$ receptors such as those related to receptor desensitization (Macdonald & Twyman, 1992; Lavoie & Twyman, 1996).

At certain synapses at which synaptic GABA concentrations may not saturate postsynaptic receptors, BDZs can significantly enhance IPSC amplitude whilst having little effect on current waveform (Frerking *et al.* 1995). The predominant effect of BDZs on GABAergic IPSCs, however, is a prolongation of time course rather than an increase in current amplitude (Segal & Barker, 1984; Vicini *et al.* 1986; Otis & Mody, 1992; Zhang, Weiner & Carlen, 1993; Mody *et al.* 1994; Poncer, Durr, Gahwiler & Thompson, 1996). This is not necessarily surprising when one considers that, following release of only one vesicle, the levels of GABA instantaneously reached in the synaptic cleft are much greater than those at which potentiation of the amplitude of GABA responses are typically produced by BDZs (Choi *et al.* 1977; Macdonald & Barker, 1978; Farrant *et al.* 1990; Colquhoun, 1992; Samson & Harris, 1992). Because transmitter concentrations in the synaptic cleft fall rapidly to basal levels following release, it seems unlikely that BDZ-induced prolongation of the IPSC can arise simply from an increased microscopic association rate for GABA.

Slowing of the decay of GABAergic IPSCs could conceivably arise from a variety of sources. These include retarded clearance of GABA from the synaptic cleft, changes in synchrony between individual release sites or slowing of $GABA_A$ receptor deactivation (Mody *et al.* 1994; Jones & Westbrook, 1995*a*, 1996; Tia *et al.* 1996*a*). In this study, concentration jump methods have been used to examine the actions of BDZs on the pre-equilibrium behaviour of $GABA_A$ receptors in murine cerebellar granule cells. The experiments indicate that BDZs both slow deactivation and speed desensitization. These two effects combined produce a previously undescribed frequency dependence in the actions of these compounds. This may have far-reaching consequences for how the function of synaptic networks are modified by BDZs. Some of these data have been presented previously in abstract form (Mellor & Randall, 1997).

METHODS

Cell culture

Postnatal day 5 C57BL/6X129/SV mice were killed by decapitation under Schedule 1 of Her Majesty's Home Office regulations on the use of animals in research. The cerebellum was rapidly removed and dissociated with trypsin (10 mg ml^{-1}) to produce granule cell-rich cultures (see Randall & Tsien, 1995). Cells were maintained in standard culture conditions (37 °C, 5% CO_2), on matrigel (Collaborative Research Inc., Bedford, MA, USA)-coated coverslips, for up to 4 weeks. The culture medium consisted of a minimal essential medium (Gibco) supplemented with 20 mM KCl, 10% (v/v) fetal calf serum (Hyclone, Logan, UT, USA), 100 mg l^{-1} transferrin (Calbiochem) and 25 mg l^{-1} insulin (Sigma). After 2 days in culture, the cells were fed with media further supplemented with 4 μM ARA-C (Sigma). Cultures were then fed every 5 days with a 50% replacement of the media. Cells used in studies of BDZ potentiation of synaptic currents were grown in 5 mM K^+, rather than 25 mM K^+, because the latter condition greatly suppresses GABAergic synaptic connectivity (Randall, Deisseroth & Tsien, 1993).

Electrophysiology

Prior to recording, a single coverslip-containing dish was removed from the incubator and the culture medium exchanged for a standard Tyrode solution consisting of (mM): 130 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 30 glucose and 25 Hepes-NaOH; pH 7·3. The coverslip was shattered and a single shard transferred to a standard

recording chamber mounted on the stage of an inverted microscope. Individual visually identified granule cells were approached with pipettes of 2–6 MΩ resistance filled with (mM): 110 CsCl, 10 NaCl, 5 $MgCl_2$, 5 EGTA, 2 ATP, 0·2 GTP and 35 Hepes-CsOH; pH 7·3. Whole-cell patch-clamp recordings were initiated by standard means. All recordings were made at room temperature (21–23 °C). Two different experimental approaches were adopted, either (i) for the collection of spontaneous synaptic currents, or (ii) for the analysis of responses to rapid applications of exogenous GABA.

To study exogenous GABA responses the recording electrode was moved away from the coverslip until a nucleated outside-out patch was formed. These patches consisted of the majority of the somatic membrane and had capacitances of ~0·5 to 1·5 pF. The nucleated patch was voltage clamped at −70 mV and placed adjacent to a theta tube, which was in turn connected to a fast piezo-electric translator (Burleigh). Different solutions flowed from either barrel of the theta tube, and the solution encountered by the patch could be rapidly switched under the control of the piezo-electric translator. Each barrel of the theta tube was fed from a six-way manifold. This allowed any one of up to six different solutions to flow through both the control and the agonist barrels. In experiments where the effects of BDZs were studied, the patch was pre-exposed to the appropriate compound(s) by including it in the solution flowing through the control barrel of the theta tube. In most experiments the GABA-containing solution in the agonist barrel of the theta tube was not supplemented with the BDZ. However, indistinguishable results were obtained in control experiments where both the agonist-containing and control solutions were supplemented with the BDZ under study.

The duration of agonist exposure (5–1500 ms) was controlled with a standard laboratory timer, which in turn was triggered by our acquisition software. Control experiments in which junctional currents were measured upon switching between normal and diluted extracellular solutions revealed that complete solution exchanges were made in ≤ 1 ms. Further evidence that the deactivation rate of the GABA response did not represent the clearance of free GABA from the vicinity of the patch was provided by much faster deactivation rates seen in the presence of either Ro15-4513 (Fig. 2) or Zn^{2+} (see Mellor & Randall, 1997). Responses to exogenous GABA applications were filtered at 2 kHz (Brownlee Precision 8-pole Bessel characteristic digital filter) and sampled at between 4 and 20 kHz using the pCLAMP 6 software suite (Axon Instruments). This software also controlled the clamp potential and was used extensively for data analysis.

The repetition rate of GABA applications was varied somewhat to account for the dose and time dependence of the desensitization process. Applications (120 ms) of 20 μM GABA (e.g. Figs 3 and 4) were separated by a 10 s period; 5 and 15 ms applications of 1 mM GABA (e.g. Fig. 2) were separated by a 30 s period; 50 ms applications of 1 mM GABA were separated by a 45 s period and longer applications of 1 mM GABA (e.g. Figs 5 and 6) were made at intervals of 1 min. Run-down or cumulative inactivation of GABA responses was rarely seen in our recordings. However, when present and greater than ~2% per agonist application recordings were discarded.

Miniature synaptic currents (mIPSCs) were measured at a holding potential of −70 mV in the maintained presence of the glutamate receptor antagonist CNQX (5 μM) and the Na^+ channel blocker TTX (1 μM). During the experiment, 10 mV step depolarizations were applied at 30 s intervals to monitor series resistance. Cells in which this increased by > 25% were discarded. Continuous records of membrane current were filtered at 50 kHz (4-pole Bessel filter) and stored on digital audio tape (Biologic). The data were subsequently replayed, low-pass filtered at 5 kHz (Digital 8-pole Bessel characteristic filter), high-pass filtered at 0·2 Hz and sampled to computer files at 10 kHz using the Fetchex program of the pCLAMP 5 suite (Axon Instruments). The resultant files were then analysed with a program that has been developed in this laboratory within the AxoBasic programming environment (Axon Instruments). This program detects synaptic events by analysing the derivative of the current record. It then produces an events list consisting of the time, amplitude, 20–80% growth time and half-width of individual synaptic currents. A second program was used to average mIPSCs for kinetic analysis. Events were aligned for averaging at the point where the derivative of their rising phase first reached a pre-set threshold. Prior to inclusion in the average each mIPSC was normalized to its peak current amplitude. Only mIPSCs not kinetically contaminated by previously or subsequently arising events were included in average responses.

Unless otherwise stated, all data are presented as means ± 1 S.E.M. Statistical testing was carried out, as appropriate, using Student's paired and unpaired t tests, or Mann–Whitney U tests. Statistical significance was deemed present at P values < 0·05. Significance levels below 0·001 are indicated as $P < 0·001$.

Fine chemicals

All recording solutions were made from analytical grade salts purchased from BDH or Fluka. Flunitrazepam (Flu), diazepam and Ro15-4513 and TTX were purchased from Research Biochemicals Inc. Ro15-1788 (Flumazenil) was a gift from F. Hoffman-La Roche of Basel. CNQX was purchased from Tocris-Cookson. The benzodiazepines were dissolved in NaOH of suitable concentration to allow the preparation of 1 or 10 mM stock solutions. CNQX was dissolved as a × 2000 stock solution in DMSO.

RESULTS

Flunitrazepam actions on miniature GABAergic IPSCs

In order to investigate the effects of BDZs on GABAergic IPSCs in granule cells we chose to study the postsynaptic responses elicited by the spontaneous release of the contents of single vesicles. Such mIPSCs first appeared after ~4 days of culture and were routinely present thereafter (see Fig. 1*A* for an example). The GABAergic identity of these events was confirmed by their reversible elimination with the $GABA_A$ receptor antagonists bicuculline methoiodide and picrotoxin (data not shown). After 14–17 days *in vitro* (DIV) the mean mIPSC frequency was 5 ± 2·1 Hz ($n = 8$). The mean mIPSC amplitude, 20–80% rise time and half-width measured from these cells are detailed in Table 1.

To analyse the kinetics of the IPSC more closely, 100 mIPSCs from each cell were averaged, and the decay phase fitted with both single and double exponential functions. Although visually quite good fits were produced with single exponential functions (mean decay time constant of 13·3 ± 1·1 ms), analysis of the variance ratios of the residuals from mono- and biexponential fits indicated that the decay of the mIPSC was significantly better fitted by a double exponential function (mean time constants, 6·8 ± 0·9 and 25·6 ± 5·7 ms). The fast time constant accounted for 49·7 ± 11·1% of the total decay (see also Table 2). These

Table 1. Effects of Flu on mIPSC properties

	Frequency (Hz)	Amplitude (pA)	20–80% rise time (μs)	Half-width (ms)
Control	5·0 ± 2·1	31·4 ± 3·1	650 ± 50	6·7 ± 0·7
Flu	6·1 ± 3·0	38·9 ± 4·6	670 ± 60	11·4 ± 1·6
P	n.s.	< 0·045	n.s.	< 0·005

The table indicates the mean frequency, amplitude, 20–80% rise time and half-width of mIPSCs recorded from seven cerebellar granule cells under control conditions and in the presence of Flu (1 μM). The cells had been in culture for 14–17 days. Note that the IPSCs that were recently preceded by another event were rejected from the half-width determinations. At least 200 events were analysed per cell. The bottom row documents significance levels in paired *t* tests, and indicates that both the mean amplitude and the half-width were significantly greater in the presence of Flu (n.s., not significantly different).

Table 2. Comparison of the kinetics of mIPSCs and responses to brief receptor-saturating applications of 1 mM GABA in the presence and absence of Flu

	Monoexponential		Biexponential			
	τ_1 (ms)	*n*	τ_1 (ms)	τ_2 (ms)	% τ_1	*n*
mIPSC	13·3 ± 1·1	8	6·8 ± 0·9	25·6 ± 5·7	49·7 ± 11·1	8
5 ms, 1 mM GABA	86 ± 7·3	37	42·0 ± 3·7	260 ± 23	72·6 ± 2·1	37
P	< 0·001	Unpaired	< 0·001	< 0·001	< 0·002	Unpaired
mIPSC, control	12·8 ± 1·1	7	7·3 ± 1·4	30·4 ± 8·7	58·5 ± 15·4	5
mIPSC + Flu	21·5 ± 1·6	7	11·2 ± 2·0	25·2 ± 2·2	35·7 ± 7·5	5
P	< 0·002	Paired	n.s.	n.s.	n.s.	Paired
5 ms, 1 mM GABA, control	88·6 ± 11·7	20	42·6 ± 5·2	267·2 ± 32·8	70·8 ± 3·4	20
5 ms, 1 mM GABA + Flu	121 ± 14	20	50·9 ± 6·6	306 ± 33	61·6 ± 2·4	20
P	< 0·001	Paired	< 0·005	n.s.	< 0·002	Paired

The table documents three kinetic comparisons. The first three rows illustrate the differences between the decay kinetics, both mono- and biexponential, of mIPSCs and responses to 5 ms applications of 1 mM GABA recorded from cerebellar granule neurones. The second three rows document the data for the kinetics of the mIPSC recorded in the presence and absence of Flu. The final three rows make similar comparisons for the responses to brief agonist applications made in the presence and absence of Flu. The column *n* indicates the number of observations included in each data set and whether paired or unpaired *t* tests were used in the statistical comparisons. τ, time constant.

values are close to those reported for IPSCs recorded from granule cells in cerebellar slices (Tia *et al.* 1996*a*).

In seven cells, application of the BDZ Flu (1 μM) produced no significant change in mIPSC frequency or rise time. In contrast, statistically significant increases in both mIPSC amplitude (Fig. 1*C*) and half-width (Fig. 1*B* and *D*) were consistently observed. The change observed in the latter parameter (1·67 ± 0·11-fold), however, was significantly greater than that seen in the former (1·24 ± 0·08-fold). The increase in mIPSC half-width produced by Flu was completely reversed by a sufficiently prolonged period of washout (Fig. 1*E*). The effects of Flu on these basic parameters of individual granule cell mIPSCs are detailed in Table 1.

In the presence of Flu, single exponential fits to the decay phase of averaged mIPSCs exhibited significantly longer time constants than in control ($P < 0·002$, paired *t* test, Table 2). The situation with biexponential fits, however, was less clear. In two of the seven cells examined the decay of the IPSC was no longer biexponential in the presence of Flu. In the remaining five cells, we observed the following non-significant trends upon addition of Flu: a decrease in the slow time constant, an increase in the fast time constant and a decrease in the overall contribution of the fast component of decay. The parameters of both biexponential and monoexponential fits to data recorded in the presence and absence of Flu are detailed in Table 2.

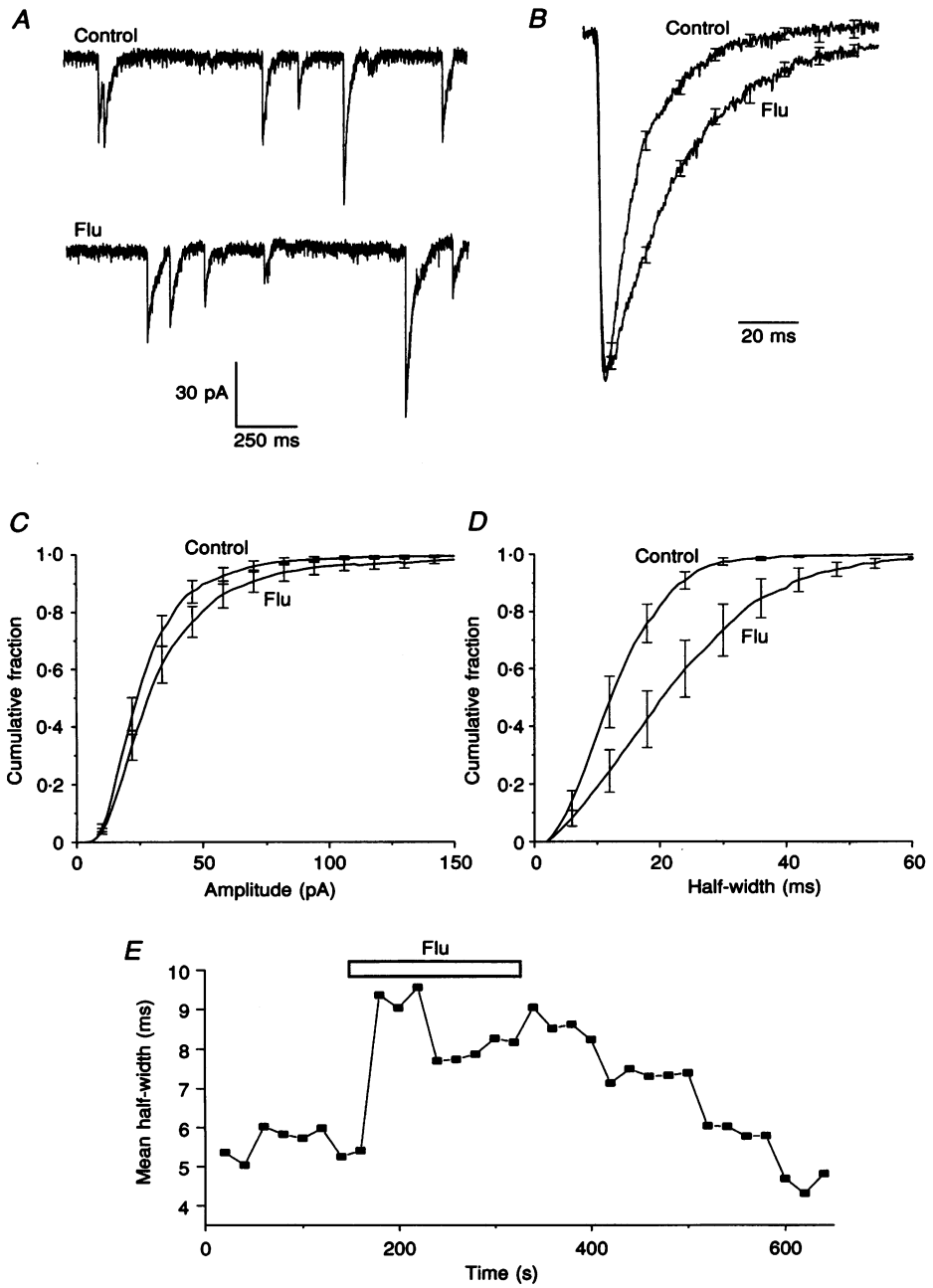

Figure 1. BDZ actions on miniature IPSCs

A, mIPSCs recorded at a holding potential of −70 mV from a cultured cerebellar granule cell in the presence of 5 μM CNQX and 1 μM TTX (top) and following the addition of 1 μM Flu (bottom). *B*, averaged mIPSCs recorded in the presence and absence of Flu. Events are from a different cell to that shown in *A*. Each average was made from twenty randomly selected mIPSCs under each condition. Each event was normalized to peak inward current before being incorporated into the average. Occasional error bars from the averaging procedure are shown. *C*, average cumulative amplitude curves derived from seven cells recorded in the presence and absence of Flu (1 μM). Between 200 and 2500 events were used to construct each curve (i.e. Control and Flu) in each cell. *D*, average cumulative half-width curves recorded in the presence (Flu) and absence (Control) of Flu (1 μM). Data from the same seven cells used for *C*. *E*, a plot of mean mIPSC half-width *versus* time from an example cell illustrates the reversibility of Flu-induced broadening of the mIPSC in cerebellar granule cells. All mIPSC half-widths in consecutive 20 s bins were averaged to produce each point shown.

Thus although decay of the mIPSC was apparently biexponential, the precise way in which these kinetics were altered by Flu to produce the significantly broadened mIPSC seemed beyond the resolution of our recordings. In the main, this was because of the relatively modest size of the mIPSC coupled with the small separation between the two time constants of decay. We therefore adopted another approach to probe how BDZs might produce their effects on GABAergic postsynaptic currents.

Flunitrazepam effects on responses to brief receptor-saturating applications of GABA

Responses produced by agonist applications designed to mimic the temporal profile of neurotransmitter in the synaptic cleft are increasingly being used to probe the synaptic consequences of changes to postsynaptic receptors (Jones & Westbrook, 1995*a*; Lavoie & Twyman, 1996; Tia *et al.* 1996*a*). We used this approach to investigate the effects of BDZs on the GABA responses in cerebellar granule cells. Responses were generated by making 5 ms step applications of 1 mM GABA to nucleated patches voltage clamped at −70 mV. Such agonist applications produced responses that rose rapidly to a peak before decaying over a period of a few 100 ms, and thus resembled IPSCs.

Kinetic and statistical analysis indicated that, like mIPSCs, responses to brief pulses of 1 mM GABA decayed (deactivated) biexponentially. The rate of decay for responses to exogenous GABA applications, however, was considerably slower than that of the mIPSC in granule cells and also involved a greater contribution of the fast component to the overall decay (Table 2).

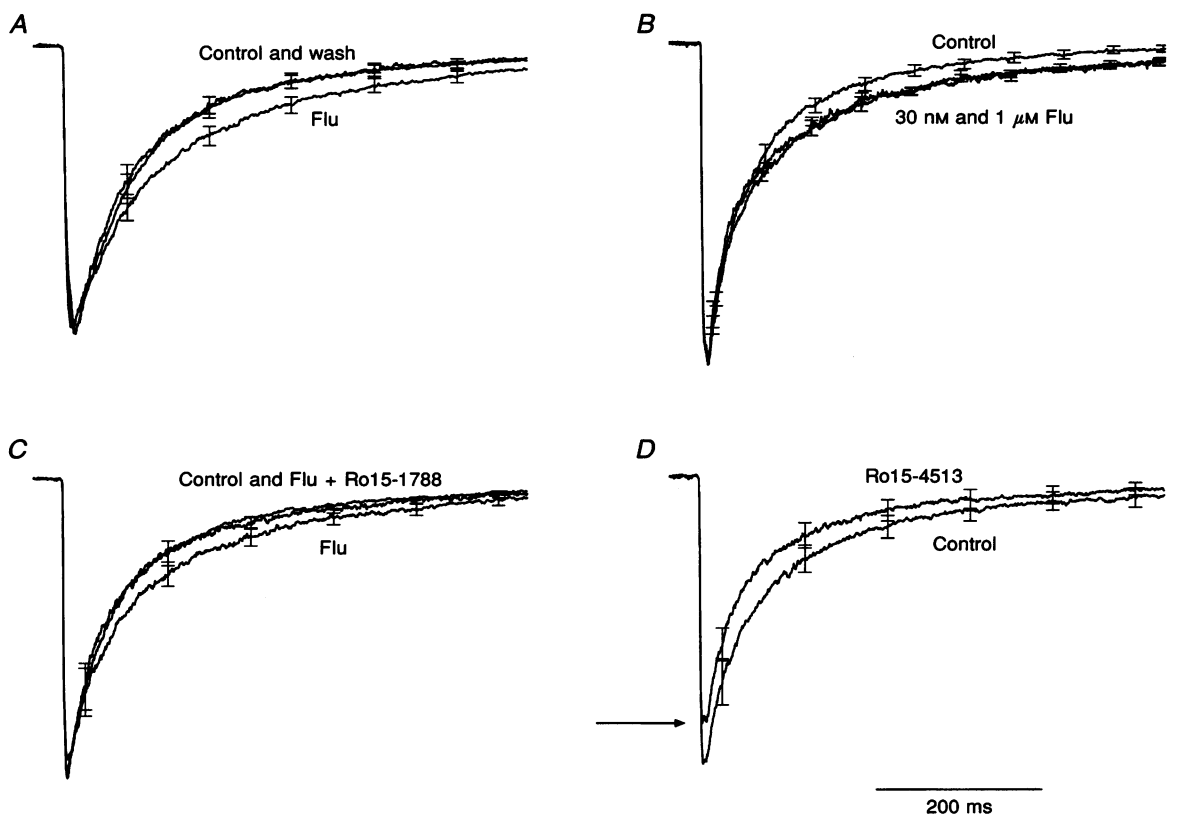

Figure 2. Actions of BDZs on brief receptor-saturating applications of GABA

A, 1 μM Flu broadens, but has no effect on the amplitude, of responses generated by 5 ms applications of 1 mM GABA. Complete reversal of the effects of Flu are seen following washout. The traces from each cell were normalized to their pre-drug amplitude before averaging across cells ($n = 10$). *B*, sweeps averaged from twelve cells illustrating that 1 μM and 30 nM Flu produce very similar effects on responses to 5 ms applications of 1 mM GABA. In each cell, the dose of Flu was increased stepwise from 30 nM to 100 nM to 300 nM to 1 μM. Response at the intermediate concentrations resembled those at 30 nM and 1 μM Flu. *C*, mean data from five cells illustrating the reversal of Flu-induced response broadening by the benzodiazepine receptor antagonist Ro15-1788 (10 μM). The experimental protocol consisted of recording control sweeps, followed by those in the presence of Flu, followed finally by those in the presence of Ro15-1788 and Flu. All currents were normalized to their pre-drug amplitude before averaging. *D*, an illustration of the speeding of deactivation and depression of peak current amplitude afforded by the partial inverse agonist Ro15-4513 (10 μM). For clarity the arrow indicates the level of the depressed peak current in the presence of the drug. Data were averaged from six cells, as described for *A*.

Figure 2*A* shows such responses averaged from ten cells before, during and following the application of 1 μM Flu. Averaging across cells was carried out after currents were normalized to the peak inward current observed in the absence of Flu. It is clear that Flu produced no change whatsoever in the mean current amplitude (101 $\pm$ 2%). However, as seen in recordings of mIPSCs (Fig. 1*B* and *D*), the duration of the GABA response was considerably greater in the presence of Flu. This increase in response width arose seemingly entirely from changes in the deactivation (i.e. falling) phase of the GABA response and could be entirely reversed by Flu washout. The slowing of deactivation led to a mean increase of 36 $\pm$ 7% ($n = 11$) in the total charge transferred per agonist application. Two of the non-

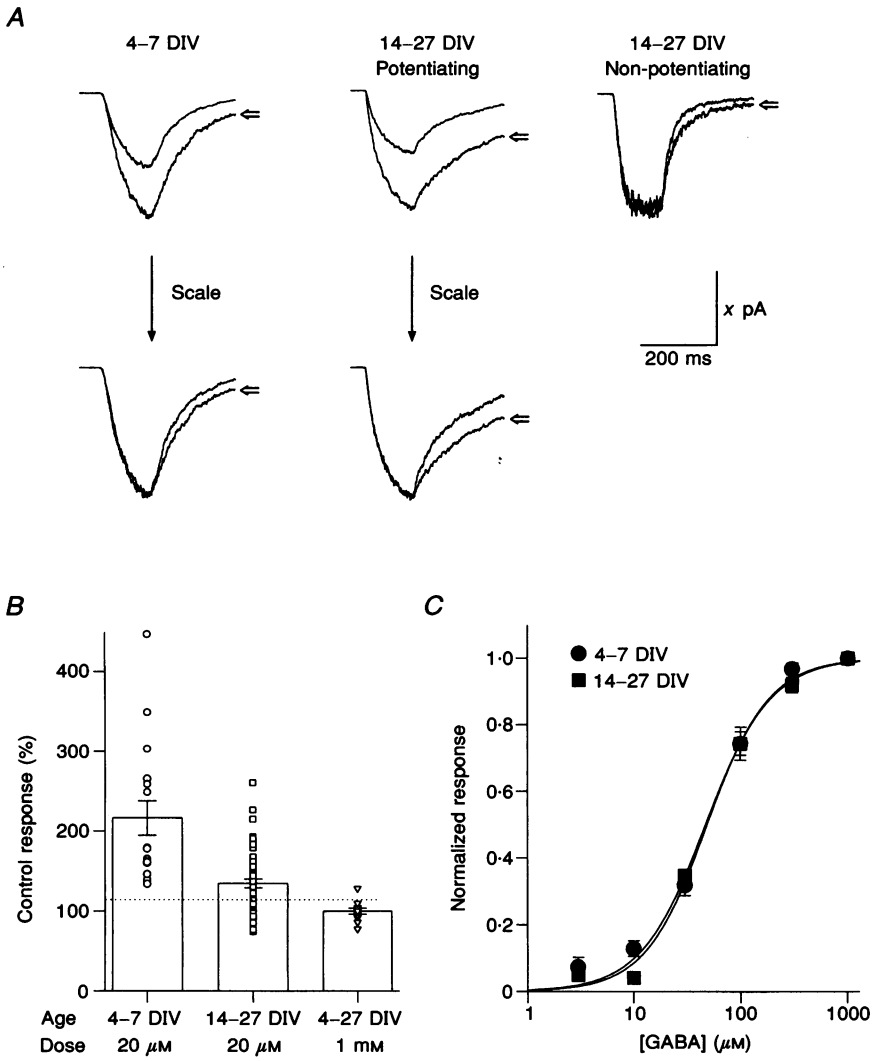

Figure 3. Changes in BDZ sensitivity with increased time in culture

A, the top row illustrates example responses to 20 μM GABA recorded in the presence (open arrow) and absence of 1 μM Flu. The individual examples depict typical cells from the following populations: left, a short-term culture; centre, a long-term culture potentiated by Flu; right, a long-term culture unpotentiated by Flu; the scale bar value x is equal to 320, 370 and 165 pA, respectively. The bottom row illustrates versions of the left and centre panels of the top row, scaled such that the peak currents in the presence and absence of Flu are identical. Note that deactivation of the current response is slowed in all three groups. *B*, graph summarizing the average percentage change in I_{GABA} produced by 1 μM Flu in the three experimental conditions indicated on the abscissa. The symbols illustrate the data for each cell tested, whereas the bars illustrate the group means and standard errors. The dotted line indicates the level (i.e. 115% of control) of the potentiation threshold we set. *C*, dose–response curves averaged from either 4–7 DIV ($n = 4$, ●) or 14–17 DIV ($n = 6$, ■) cerebellar granule cells. Currents were elicited with 120 ms applications of 3, 10, 30, 100, 300 and 1000 μM GABA. Prior to averaging, current amplitudes at each dose were normalized to the peak current amplitude measured at 1 mM GABA. The lines represent the best logistic fits to the data. These had EC_{50} values of 47·1 μM (4–7 DIV) and 48·7 μM (14–17 DIV); the Hill coefficients were 1·43 and 1·50, respectively.

significant trends in mIPSC kinetics seen upon the addition of Flu (Table 2) were reproduced in the responses to exogenous GABA. In this case, however, the increase in the fast time constant and the greater contribution of the slow time constant were statistically significant (Table 2).

Very similar observations were made when another BDZ agonist (diazepam, 1 μM) was used in place of Flu ($n = 4$, data not shown). Although most of our experiments utilized 1 μM Flu, preliminary investigation of the dose dependence of the actions of this drug indicated that much lower concentrations were sufficient to produce maximum activity. Indeed, as illustrated in Fig. 2*B*, the broadening of the response to 1 mM GABA was as great with 30 nM Flu as with 1 μM Flu ($n = 12$).

We tested whether the Flu-induced changes in GABA responses shown in Fig. 2*A* and *B* arose from some non-specific activity unrelated to occupancy of the BDZ site. To do this, the BDZ site antagonist Ro15-1788 (Flumazenil, 10 μM) was used. Application of this compound completely reversed the actions of Flu (Fig. 2*C*, $n = 5$). The compound Ro15-4513, a partial inverse agonist at the BDZ site, produced kinetic actions opposite to those of Flu. Thus, commensurate with its activity on GABAergic synaptic transmission (Krespan, Springfield, Haas & Geller, 1984; King, Knox & Dingledine, 1985; Vicini *et al.* 1986), Ro15-4513 considerably speeded the deactivation of responses to 5 ms applications of 1 mM GABA, and also produced a $23 \pm 12\%$ decrease in their amplitude (Fig. 2*D*, $n = 6$). The control current phenotype was consistently recovered upon washout of Ro15-4513 (data not shown).

It was therefore possible to demonstrate similar effects of BDZs on currents produced by either synaptically released GABA or exogenously applied GABA. An experimental shortcoming of studying synaptic currents is that it is very difficult to reliably change either the concentration or synaptic lifetime of released transmitter. Because this is not a problem when exogenous GABA is applied, this latter method was used to investigate further the effects of Flu on non-steady-state features of GABA responses.

Age-dependent effects of Flu on currents elicited by GABA

As recently reported elsewhere (Jones *et al.* 1997), Flu fails to potentiate the amplitude of responses to submaximal concentrations of GABA in a significant fraction of cerebellar granule cells maintained in culture for > 14 days. This contrasts with the observation that Flu broadened mIPSCs in all cells cultured for a similar period ($n = 7$, see Fig. 1). Some considerable differences were found when the actions of Flu on young (4–7 DIV) and old (14–27 DIV) cultures of cerebellar granule cells were compared. The peak amplitude of the response produced by a 120 ms application of 20 μM GABA (I_{GABA}) was potentiated in all cells taken from young cultures ($n = 15$, Fig. 3*A* and *B*). In contrast, Flu potentiated I_{GABA} in only ~55% (30 of 55) of cells taken from older cultures (Fig. 3*A* and *B*). As would be predicted from previous work (Choi *et al.* 1977; Macdonald & Barker, 1978; Farrant *et al.* 1990), and irrespective of whether the cells were obtained from young or old cultures, the amplitude of responses elicited by 120 ms applications of 1 mM GABA was unaffected by Flu ($100 \pm 4\%$ of control, Fig. 3*B*).

Pooled dose–response curves determined with 120 ms GABA applications exhibited EC_{50} values close to 45 μM. The value of the EC_{50} was not significantly different when cells taken from young and old cultures were compared (Fig. 3*C*). Furthermore, the EC_{50} of neurones in which I_{GABA} was potentiated by Flu was not significantly different from the EC_{50} of neurones in which I_{GABA} was unpotentiated by Flu (data not shown). This means an increased affinity for GABA cannot explain the delayed appearance of a population of cells in which Flu is incapable of increasing the amplitude of responses to 20 μM GABA.

An additional difference between cells with Flu-sensitive and Flu-insensitive I_{GABA} was in the activation kinetics of the GABA response. Statistical analysis of responses to 20 μM GABA revealed that cells exhibiting response amplitude potentiation in the presence of Flu had significantly slower ($P < 0.01$, unpaired t test) current activation rates ($\tau_{act} = 49.6 \pm 4.0$ ms, $n = 46$) than cells in which Flu produced no increase in response amplitude ($\tau_{act} = 28.0 \pm 2.0$ ms, $n = 25$).

Effects of Flu on kinetics of GABA responses

The most widely accepted view of the mechanism of action of BDZs is that they facilitate the binding rate of the first GABA molecule to the GABA receptor complex (Macdonald & Twyman, 1992; Rogers *et al.* 1994; Lavoie & Twyman, 1996). Under most conditions this would be expected to lead to a speeding of the rising phase of GABA responses. Somewhat surprisingly, and in contrast to another recent study (Lavoie & Twyman, 1996), the mean activation time constant of currents produced by concentration jumps to 20 μM GABA was significantly increased in the presence of 1 μM Flu (control, 39.8 ± 2.8 ms *vs.* Flu, 45.7 ± 2.9 ms; $n = 73$).

A more striking kinetic effect produced by Flu was a slowing of the rate of deactivation. This could be conveniently visualized by scaling the GABA response produced in the absence of the BDZ to match that recorded in the presence of the BDZ (Fig. 3*A*). To compare the slowing of deactivation observed in different experimental groups, single exponentials were fitted to the deactivation trajectory of GABA responses elicited before, during and following the application of Flu (1 μM). Normalized averaged plots of such analyses are shown in Fig. 4*A*. In sufficiently long-lasting recordings, complete recovery of the deactivation rate to control values was observed. This typically took a washout period of ~300 s (data not shown).

The top panels of Fig. 4*A* make it clear that Flu produced an approximately similar slowing of deactivation in young and old granule cell cultures, even though only 55% of older

cells responded to BDZ application with an increase in the amplitude of their GABA response (Fig. 3*B*). To investigate this apparent discrepancy further, the granule cells of older cultures were separated into two groups: those in which Flu potentiated the amplitude of the GABA response, and those in which Flu did not. The increases in deactivation time constant produced by Flu in each group were statistically indistinguishable, averaging 1·68 ± 0·07- and 1·55 ± 0·07-fold, respectively (Fig. 4*A*, bottom). It seems, therefore, that at both submaximal and supermaximal GABA concentrations, Flu can produce effects on GABA response deactivation in the absence of effects on response amplitude.

Although in Fig. 4*A* a single exponential fit was used to produce a useful empirical estimate of deactivation rate, in reality deactivation of responses to 20 μM GABA in cultured granule cells were, like those to 1 mM GABA, consistently biexponential (Fig. 4*B*; see also Jones & Westbrook, 1995*a*; Tia *et al.* 1996*a*). These time constants averaged 40·4 ± 2·1 and 251 ± 15 ms (Fig. 4*C*, filled bars). In contrast to the

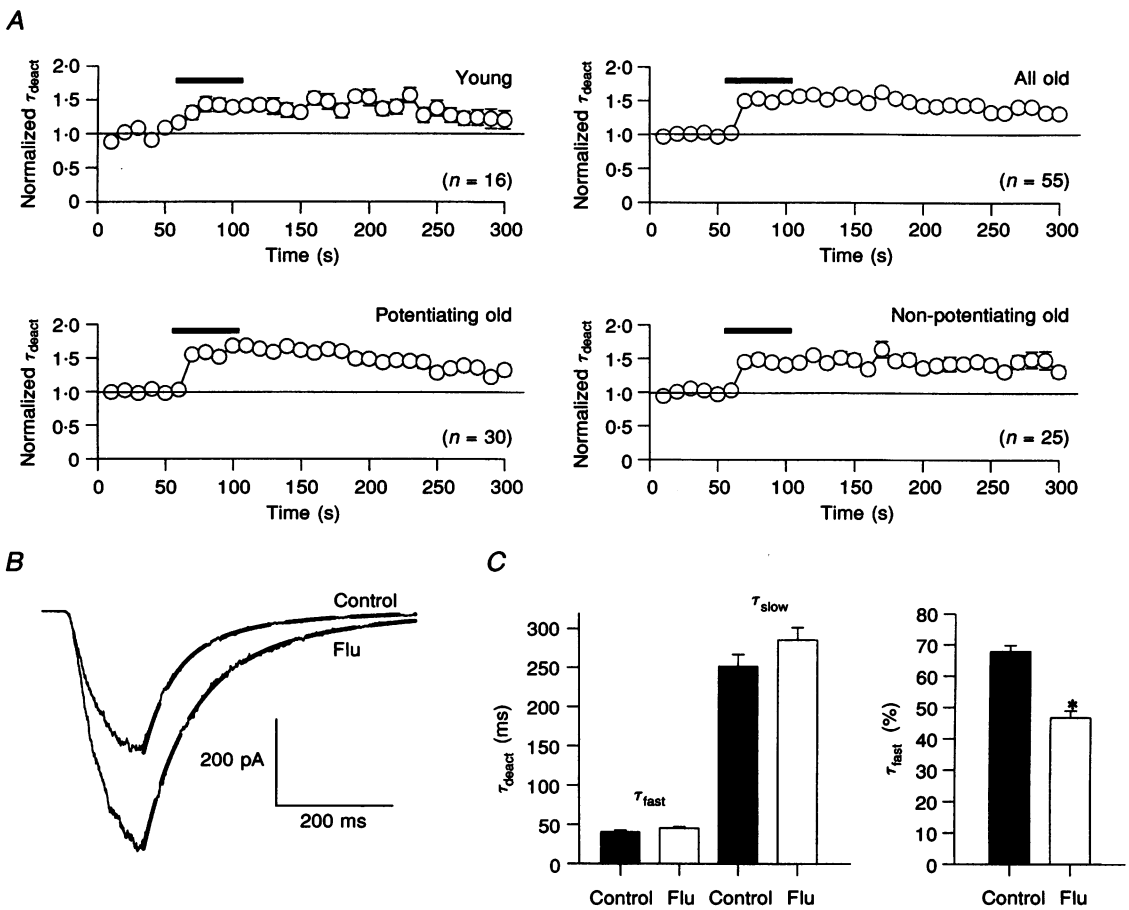

Figure 4. Flu slows current deactivation irrespective of whether it potentiates current amplitude
A, graphs plotting the time course of the time constant of the best single exponential fit to the deactivation trajectory of the response generated by 20 μM GABA, before, during, and following the application of Flu (1 μM, indicated by the horizontal bar). Before averaging across the relevant population of cells, deactivation rates from each neurone at each time point were normalized to the mean deactivation rate prior to Flu application. Error bars from the averaging process are shown when larger than the symbol. The individual graphs represent data from the following: top left, all cells from young cultures (i.e. 4–7 DIV, $n = 16$); top right, all cells from mature cultures (i.e. 14–27 DIV, $n = 55$); bottom left, cells from mature cultures exhibiting > 15% potentiation of I_{GABA} by Flu ($n = 30$); bottom right, cells from mature cultures in which I_{GABA} was not potentiated by Flu ($n = 25$). *B*, example currents elicited by 20 μM GABA in the presence and absence of Flu (1 μM) illustrating biexponential fits (dashed lines) to the deactivation trajectory of the response. The curves shown represent the functions $I = -230\exp(-t/48) + -108\exp(-t/174)$ (Control) and $I = -341\exp(-t/66) + -226\exp(-t/205)$ (Flu), where t is the time elapsed since removal of GABA and I is the current remaining. *C*, histograms presenting the effects of Flu (1 μM) on the biexponential deactivation trajectory of GABA responses. Left, a histogram showing that Flu has no significant effect on either time constant of deactivation (τ_{deact}). Right, a histogram illustrating that Flu increases the fraction of the total deactivation mediated by the slow time constant. The asterisk indicates statistical significance.

results obtained with 5 ms applications of 1 mM GABA, Flu produced no significant change in either deactivation time constant of responses to 20 μM GABA (time constants in Flu, 45·1 ± 2·1 and 285 ± 16 ms). Under these conditions the observed slowing of deactivation seemed to arise solely from a significant increase in the contribution of the slow time constant to the overall decay (Fig. 4*C*, right).

Coupling of desensitization and deactivation in granule cells

Mechanistically, the effects of Flu on deactivation shown in Fig. 4 are reminiscent of the slowing of deactivation of GABA responses produced by increased desensitization in hippocampal neurones (Jones & Westbrook, 1995*a*, 1996). Figure 5 illustrates that a similar coupling of deactivation

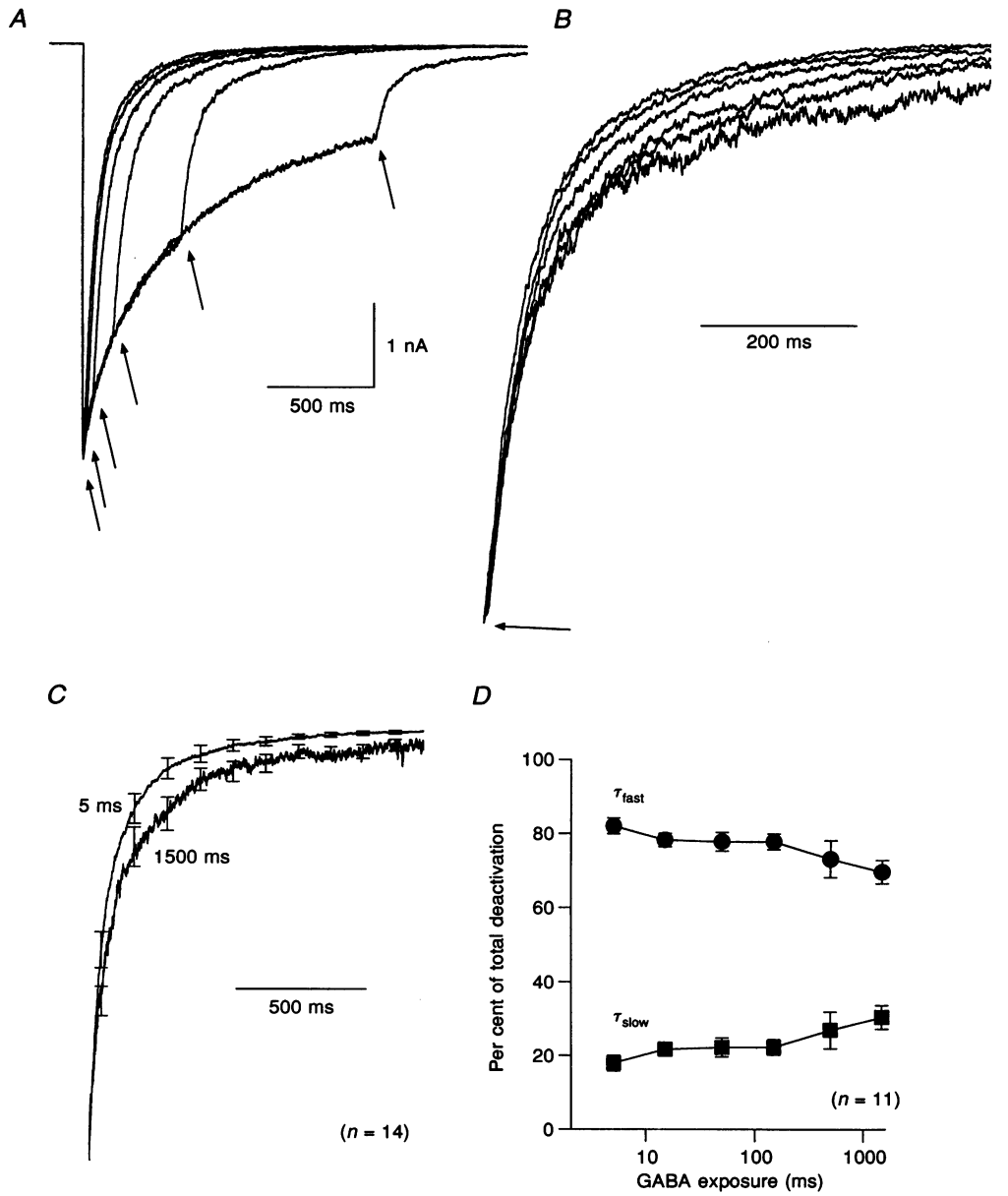

Figure 5. Desensitized states prolong deactivation of GABA responses

A, sweeps from an example cell generated by 5, 15, 50, 150, 500 and 1500 ms applications of 1 mM GABA. *B*, a comparison for the cell in *A* of the deactivation trajectories following each duration of agonist exposure. For comparison, data were normalized to the current level at the time of agonist removal (arrows in *A*), which was also set as time zero (arrow in *B*). The order of deactivation rate mirrors the duration of agonist exposure, with the shortest exposures exhibiting the fastest deactivation. *C*, pooled data from fourteen cells illustrating the differences in deactivation rate following 5 and 1500 ms applications of 1 mM GABA. Before averaging, data were normalized as in *B*. *D*, graph plotting the percentage of the total decay mediated by the fast and slow components of deactivation following a range of durations of exposure to 1 mM GABA. Data from eleven cells.

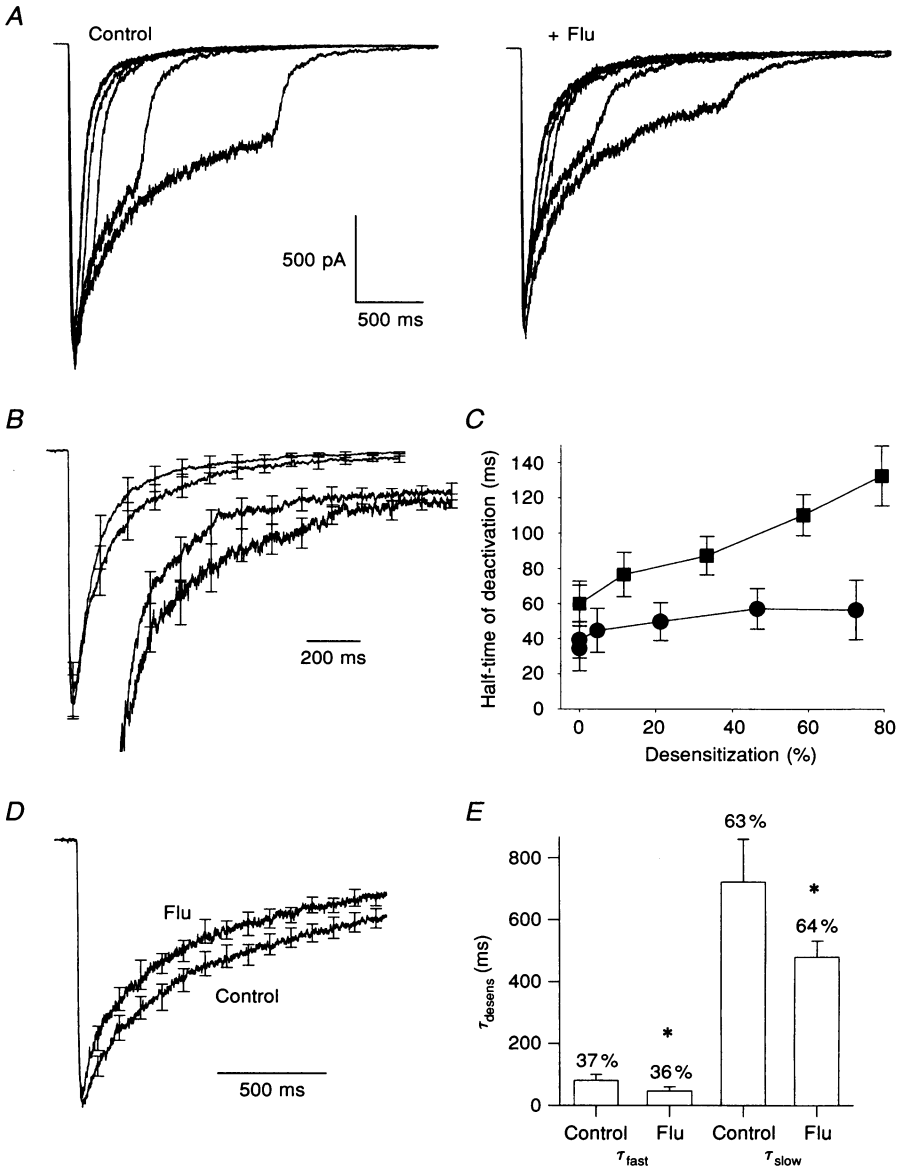

Figure 6. Prior desensitization fails to reverse the actions of Flu

A, data from an example cell illustrating responses to 5, 15, 50, 150, 500 and 1500 ms applications of 1 mM GABA in the absence (left) and presence (right) of 1 μM Flu. *B*, average responses, recorded in both the presence and absence of Flu (1 μM), to 5 (left) and 1500 ms (right) applications of 1 mM GABA. Before averaging, sweeps from each of six cells were normalized to the current amplitude immediately prior to GABA removal. The more slowly deactivating traces are those recorded in the presence of Flu. *C*, graph plotting, for five experiments like that in *A*, the average half-time of deactivation *versus* the level of macroscopic desensitization observed prior to agonist removal. The most leftward symbols arise from the briefest agonist applications, the more rightward from their more prolonged counterparts. Data are shown for sweeps recorded in both the absence (●) and presence (■) of Flu (1 μM). *D*, average macroscopic desensitization trajectories, in the presence and absence of Flu (1 μM), for 1500 ms applications of 1 mM GABA. Sweeps from each cell were normalized to peak current before averaging across all cells tested ($n = 7$). *E*, histogram plotting the values of the two time constants of desensitization (τ_{desens}) for six cells recorded in the presence (Flu) and absence (Control) of Flu (1 μM). The percentages above each bar indicate the contribution to the total desensitization made by each time constant. The percentage decrease of τ_{fast} and τ_{slow} was 27 ± 19 and $26 \pm 11\%$, respectively. The asterisks indicate significant differences in the desensitization time constants when comparisons of sweeps made under control conditions and in the presence of Flu are compared.

and desensitization is present in cultured cerebellar granule cells. Prolonged exposure to 1 mM GABA produced substantial macroscopic desensitization of GABA responses in all granule cells examined (Fig. 5*A*). Good fits to the macroscopic desensitization trajectory produced with 1·5 s applications of GABA required two time constants of 47 ± 11 and 479 ± 49 ms ($n = 7$; see also Cash & Subbarao, 1987; Celentano & Wong, 1994; Gingrich, Roberts & Kass, 1995; Jones & Westbrook, 1995*a*, 1996). Analysis of the currents produced with different periods of exposure to 1 mM GABA clearly indicated that deactivation rate was dependent on the length of prior agonist exposure. This was best visualized if, prior to alignment to compare deactivation trajectories, individual sweeps were normalized to their current amplitude immediately prior to agonist removal (Fig. 5*B*). Pooled data ($n = 14$) treated in a similar fashion (Fig. 5*C*) show the marked change in deactivation rate when 5 and 1500 ms agonist exposures are compared.

The slowing of deactivation produced by prolonged agonist exposure was quantified by fitting double exponentials to the deactivation time course. For all durations of GABA

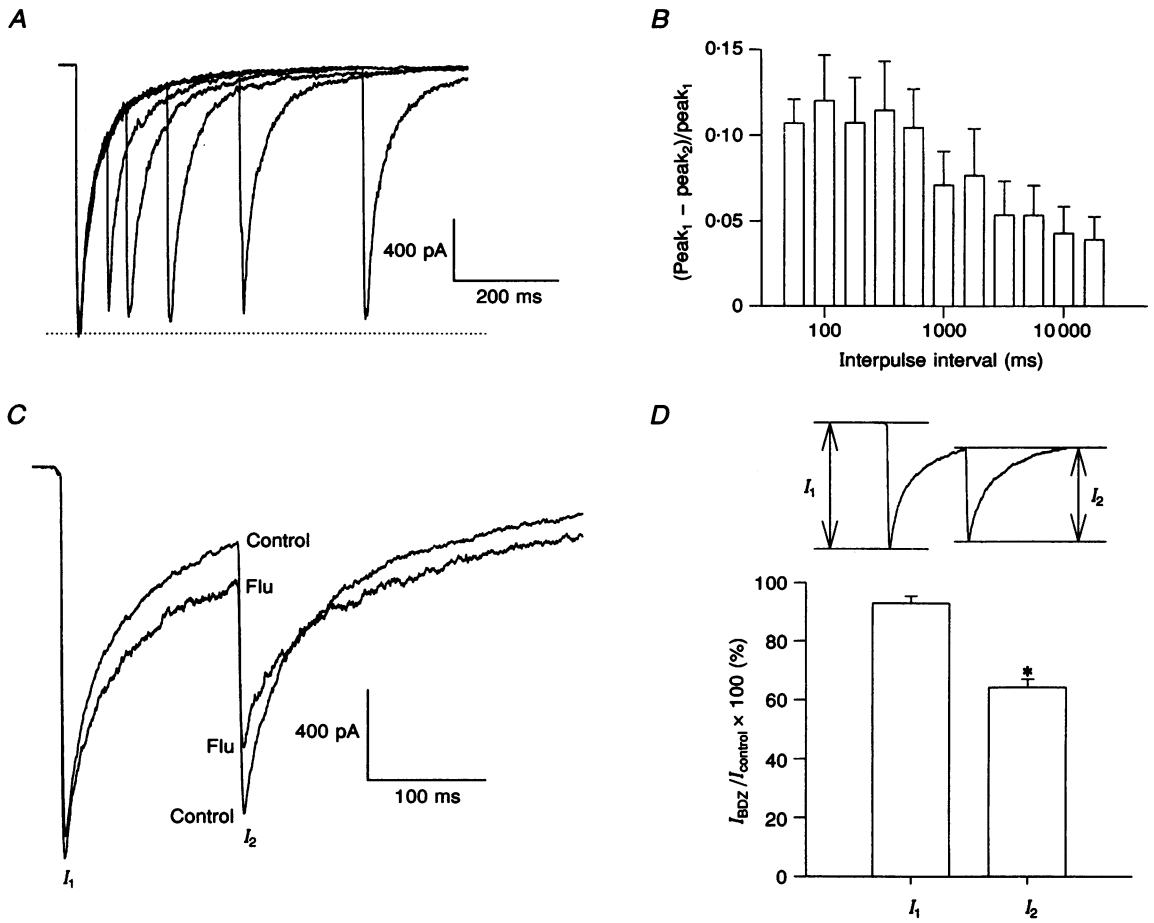

Figure 7. The effect of BDZs on $GABA_A$ receptors can reflect recent receptor activity

A, data from an example experiment illustrating the generation of slowly recovering desensitized channels with a single 5 ms, 1 mM GABA application. A first 5 ms application of 1 mM GABA was made soon after the onset of the sweep and was followed at a variable latency by a second identical application. The dotted line indicates the average level of the first response. *B*, histogram plotting the fractional depression of the peak amplitude of the second GABA response with respect to the first from paired application protocols similar to that shown in *A*. Note the slow recovery of the second response as the interpulse interval is increased. Data averaged from fifteen cells. The peak current amplitude of the first response ($peak_1$) and the second response ($peak_2$) were both measured from the baseline current prior to the first agonist application. *C*, paired responses generated by two successive 5 ms applications of 1 mM GABA. The interval between GABA applications was 150 ms. Example responses recorded in the presence (Flu) and absence (Control) of Flu (1 μM) are shown. Note that although the amplitude of the current generated by the first application of GABA is little affected by Flu, the amplitude of the second IPSC is considerably depressed in the presence of this BDZ. *D*, the histogram shows the effect of Flu (1 μM) on the amplitude of the first (I_1) and second (I_2) current responses. Data are pooled from thirteen experiments like that shown in *A*. The top panel illustrates how I_1 and I_2 were measured. The asterisk indicates a significant difference between the effects of Flu on I_1 and I_2.

application tested, neither the fast nor the slow time constant of deactivation was significantly different from that recorded following a 5 ms GABA application (data not shown). The longer agonist exposures did, however, increase the fraction of the total deactivation process mediated by the slower of the two time constants (Fig. 5*D*). These observations are in good agreement with those of Jones & Westbrook (1995*a*, *b*).

Investigation of the interplay between desensitization and the actions of flunitrazepam

Thus both an increase in desensitization and application of Flu produce similar kinetic effects on deactivation of $GABA_A$ receptors. We therefore investigated whether the degree of desensitization induced before agonist removal altered the ability of Flu to slow deactivation. To do this, we compared the deactivation of responses to different durations of GABA application, both in the presence and absence of Flu (1 μM). Sweeps from an example experiment are shown in Fig. 6*A* and pooled data from six such experiments illustrating the time course of the deactivation process following 5 and 1500 ms agonist exposures are shown in Fig. 6*B*. It is clear that deactivation was slowed by Flu, irrespective of the duration of the prior GABA application.

These experiments were quantified by analysing the time required for deactivation to reach 50% completion following different durations of agonist exposure made in both the presence and absence of Flu. The results of this analysis are plotted as percentage macroscopic desensitization *versus* deactivation half-time, and confirm that Flu slowed deactivation over a wide range of agonist exposure durations and levels of receptor desensitization (Fig. 6*C*). Calculation of the change in deactivation half-time produced by Flu for each period of agonist exposure revealed a non-significant trend ($P < 0{\cdot}07$) towards a greater Flu-induced slowing of deactivation under more desensitized conditions.

Thus prior desensitization of $GABA_A$ receptors does nothing to compromise the effects of Flu on deactivation rate. Indeed, slowing of deactivation by this BDZ may occur to a somewhat greater degree following long agonist exposures. This could be explained if Flu itself was able to enhance desensitization. Comparison of the macroscopic desensitization trajectories of responses to 1500 ms applications of 1 mM GABA unequivocally demonstrated that Flu enhances the rate of desensitization (Fig. 6*D*). Kinetic analysis of this effect revealed that the BDZ decreased both desensitization time constants, without altering the fractional contribution of each component to the total decay (Fig. 6*E*). Washout of Flu produced complete recovery to the control desensitization trajectory.

Frequency-dependent actions of Flu

It has been clearly demonstrated that even brief exposure to synaptically relevant concentrations of GABA can produce a degree of $GABA_A$ receptor desensitization (Jones & Westbrook, 1995*a*; Tia *et al.* 1996*a*, *b*). The induction of desensitization by brief agonist pulses can be most conveniently demonstrated using paired agonist application protocols. An example of such an experiment is shown in Fig. 7*A*. Here, a first 5 ms application of 1 mM GABA was followed, with a variable latency, by a second identical agonist application. This resulted in a second response that was somewhat smaller than the first. The degree to which the second response was depressed reflects the combination of the amount of receptor desensitization produced during the first 5 ms GABA application, and the degree of recovery from desensitization achieved between the two applications. This effect is quantified for experiments performed on fifteen cells in Fig. 7*B*. The histogram shows the depression of the second response as a fraction of the amplitude of the first response (note the logarithmic time axis). It is clear that the second response was detectably smaller with inter-stimulus intervals as long as 20 s.

To investigate how the combined effects of BDZs on $GABA_A$ receptor desensitization and deactivation might affect synapses functioning at a defined afferent frequency, the actions of Flu on paired GABA responses elicited at a fixed interstimulus interval of 150 ms were investigated ($n = 13$). This interval was chosen as it represents the peak-to-peak time of the theta rhythm, an important central nervous system process in which $GABA_A$ receptors are strongly implicated. As expected from the earlier observations (Fig. 2*A*), Flu had little effect on the amplitude of the first GABA response (I_1). In contrast, the amplitude of the second GABA response (I_2) was greatly depressed in all cells tested ($n = 13$; Fig. 7*C*). This implies that Flu increased the desensitization produced during the first agonist exposure. The mean changes, produced by Flu, in I_1 and I_2 relative to control are shown in Fig. 7*D*. The effects of Flu were reversed following washout (data not shown).

The total charge transferred during each of the two GABA responses not only depends on the response amplitude, but also involves the deactivation rate. In the presence of Flu, although the amplitude of I_2 was considerably depressed, its deactivation rate, like that of I_1, was greatly slowed. The depression of peak amplitude and slowing of deactivation combine in such a way that the charge transferred in the second response was essentially unchanged by Flu. This stands in contrast to the ~30% increase in the area of first response produced in the presence of the BDZ.

DISCUSSION

Our investigation of the actions of Flu on GABA responses in cerebellar granule cells has demonstrated that this compound can produce a range of activities. In addition to the classical observation of increased current amplitude at submaximal agonist doses (Fig. 3), the experiments indicate that Flu can (i) retard deactivation following both sub- and supermaximal agonist applications, (ii) slightly slow (~15%) activation rate at submaximal agonist doses, and (iii) enhance desensitization.

These observations provide a number of novel insights into the functional activity of benzodiazepines and demonstrate a mechanism, namely slowing of deactivation, by which BDZs can alter GABA responses produced at synaptically relevant agonist concentrations (Colquhoun, 1992; Samson & Harris, 1992). Slowing of $GABA_A$ receptor deactivation is compatible with the broadening of the IPSC produced by BDZs at many CNS synapses, including those of murine cerebellar granule cells (Fig. 1). The slight slowing of current activation rate observed in the presence of Flu is also compatible with reports of BDZ actions on at least one synaptic input (Otis & Mody, 1992).

The use of brief saturating GABA applications to granule cell patches provides a convenient reductionist high signal-to-noise method with which to address questions of postsynaptic responsivity. An apparent flaw in the use of this method in our experimental system is that the deactivation kinetics of the responses to exogenous GABA deviate quite considerably from the decay kinetics of the granule cell mIPSC. Such behaviour has also been seen to a lesser degree in other systems (e.g. Tia *et al.* 1996*a*).

The decay kinetics of our responses to exogenous GABA are not determined by ineffective clearance of extracellular agonist, because much faster deactivation can be observed when the responses are modulated by Zn^{2+} (Mellor & Randall, 1997) or Ro15-4513 (Fig. 2*D*). What is more likely is that the somatic and synaptic receptors are in some way different. The most likely source of this difference is in subunit composition; however, interactions with other proteins or receptor phosphorylation (Jones & Westbrook, 1995*b*) could also play a role. A piece of evidence supporting regional heterogeneity of $GABA_A$ receptors in granule cell membranes of cerebellar cultures is our observation that those at the soma but not those at the synapse are powerfully modulated by Zn^{2+} (J. R. Mellor & A. D. Randall, unpublished observations).

It therefore seems that it is not safe to conclude that the $GABA_A$ receptors on the cell body of granule cells provide a watertight model of their counterparts at the synapse. However, as far as we can tell with the currently available data, the overall effects of Flu at the cell body and synapse seem similar (Table 2). The somatic $GABA_A$ receptors in granule cells exhibit gating kinetics very similar to the synaptic $GABA_A$ receptors at the BDZ-sensitive synapses of other cells (e.g. cultured hippocampal neurones, Jones & Westbrook, 1996*a*), and as such may provide a good model of the synaptic $GABA_A$ receptors of other systems.

Of note is that the degree of paired pulse depression illustrated in Fig. 7 is considerably less than that reported by Tia and colleagues (Tia *et al.* 1996*a*), whose exogenous agonist responses also more closely mirrored the rapid kinetics of the granule cell IPSC. The fast component of deactivation, and by analogy the fast time constant of IPSC decay, have been suggested to reflect entry into a desensitized state (Jones & Westbrook, 1995*a*). It is therefore likely that the GABAergic synapses of granule cells possess more rapidly desensitizing $GABA_A$ receptors than those at the cell body. An anomaly in this regard is that although BDZs speed desensitization (Figs 6 and 7), they also slow the fast component of deactivation (Table 2), an observation supported by the (admittedly non-significant) effects of Flu on the decay kinetics of the mIPSC.

Our data stand at odds with another recent study (Lavoie & Twyman, 1996), which has addressed similar questions using a recombinant $GABA_A$ receptor expressed in HEK-293 cells. In this study, which used the $\alpha_2\beta_1\gamma_2$ subunits (a combination unlikely to be widespread *in vivo*; Wisden, Laurie, Monyer & Seeburg, 1992), the BDZ diazepam induced both a speeding of current activation at submaximal agonist doses and a strong enhancement of the amplitude of currents elicited by 1 mM GABA. In contradiction to our observations, these workers also report that deactivation following brief agonist pulses was unaltered by diazepam. Their data, in contrast to ours, provide support for the conventional view that BDZs facilitate GABA association (Macdonald & Twyman, 1992; Rogers *et al.* 1994).

The differences between the results of our study and that of Lavoie & Twyman (1996) are unlikely to result from the use of recombinant channels *per se*. We have seen similar effects of Flu to those we report here on recombinant $GABA_A$ receptors of subunit composition $\alpha_1\beta_2\gamma_2$ (J. R. Mellor & A. D. Randall, unpublished observations). Pharmacologically, we have reproduced many of our effects of Flu using diazepam, the agonist used by Lavoie & Twyman. Indeed, to explain the apparent contradictions it is difficult not to recourse to the argument that the binding of BDZs to distinct $GABA_A$ receptors may well produce different molecular effects (e.g. potentiation of agonist binding or inhibition of agonist unbinding). Such a hypothesis is supported by our observation that the actions of Flu on granule cells change with maturation *in vitro* (Figs 3 and 4), presumably as a result of changes in the subunit complement of the $GABA_A$ receptors present (Gao & Fritschy, 1995; Lin & Bulleit, 1996).

The biophysical and pharmacological changes produced as the cells aged in culture (Figs 3 and 4) also indicate that the slowing of deactivation and the potentiation of current amplitude produced by Flu may be experimentally separable phenomena. Mechanistically, in the absence of effects arising through the desensitization process (Jones & Westbrook, 1995*a*), Flu-induced slowing of deactivation (and the potentiation of current amplitude at submaximal agonist doses) could arise from either changes in ligand unbinding or changes in channel closing rates (Lavoie & Twyman, 1996). The latter hypothesis, however, is not consistent with the effects of BDZs observed at the single-channel level (Vicini *et al.* 1987; Macdonald & Twyman, 1992; Rogers *et al.* 1994).

Our data support the hypothesis that both the classical steady-state effects of BDZs (Vicini *et al.* 1987; Macdonald & Twyman, 1992; Rogers *et al.* 1994) and the effects on the kinetics of deactivation arise from a slowing of ligand unbinding. Alterations in this parameter alone, however, cannot account for all of our observations, particularly those related to the onset of desensitization, which was clearly enhanced by Flu (Figs 6 and 7). A similar speeding of desensitization in the presence of a BDZ was recently reported elsewhere (Lavoie & Twyman, 1996).

Since desensitization itself slows deactivation (Jones & Westbrook, 1995*a*; Fig. 5), we considered whether the effects on deactivation produced by Flu arose entirely through enhanced desensitization. This seems highly unlikely for a number of reasons, one of which being that the degree of slowing of deactivation produced by Flu has little dependence on the agonist dose or duration of agonist application. Indeed, there was no significant relationship between the effects of Flu on deactivation and the level of macroscopic desensitization produced before agonist removal (Fig. 6). Furthermore, the slowing of deactivation of responses to 20 μM GABA produced by Flu is comparable to that elicited by the desensitization afforded by a 1·5 s application of 1 mM GABA. Paired agonist applications indicate that this level of desensitization is never produced by a 120 ms pulse of 20 μM GABA applied in either the presence or absence of Flu (J. R. Mellor & A. D. Randall, unpublished observations).

As recently reviewed by Jones & Westbrook (1996), interplay between activation/deactivation and desensitization of postsynaptic receptor channels can endow synaptic responses with complex activity-dependent profiles. The combination of slowing of deactivation and enhancement of desensitization produced by Flu in this study suggests that the activity of BDZs can exhibit an interesting and hitherto unappreciated complexity. For example, if postsynaptic $GABA_A$ receptors have been recently active in the presence of a BDZ, the net effect of BDZs on the amplitude of subsequently produced IPSCs is to depress rather than, as one might expect, to potentiate (Fig. 7*C* and *D*). Thus the actions of BDZs on inhibitory synaptic responses are likely to be dependent on the frequency of afferent stimulation. In this way these compounds may endow inhibitory synapses with a further component of use dependence over and above that produced by the multifaceted effects of receptor desensitization (Jones & Westbrook, 1996) and presynaptic auto-inhibition (Davies & Collingridge, 1996).

If, physiologically, all functions of the $GABA_A$ receptor were to be generated through discrete postsynaptic events (i.e. IPSPs) arising from transiently saturated postsynaptic receptors, then the slowing of deactivation and speeding of desensitization of postsynaptic $GABA_A$ receptors would presumably be the major mediators of BDZ actions *in vivo*. It is noteworthy that, due to the passive electrical properties of membranes, slowing of $GABA_A$ receptor deactivation is also likely to produce some enhancement of the amplitude of the IPSP (Poncer *et al.* 1996).

There is increasing evidence, however, that at least some of the plethora of extrasynaptic $GABA_A$ receptors are activated physiologically, either by ambient levels of extracellular GABA or by GABA 'spilled-over' from active synapses (Mody *et al.* 1994). In comparison with their synaptic counterparts, extrasynaptic $GABA_A$ receptors will experience lower concentrations of GABA for longer periods. Thus the steady-state actions of BDZs on GABA affinity (Choi *et al.* 1977; Costa & Guidotti, 1979; Study & Barker, 1981) are more likely to be relevant at extrasynaptic sites where they could increase tonic background GABA currents and/or shunts.

The spectrum of *in vivo* effects of BDZs varies between being predominantly anxiolytic, sedative, anaesthetic or anti-convulsant (Bellantuono *et al.* 1980; Hobbs *et al.* 1996). It is tempting to suggest that the relative ability of any compound to either alter GABA binding (either association or dissociation) or to facilitate receptor desensitization may shape its particular *in vivo* activity profile. To this end, we are currently investigating the kinetic effects of a range of BDZ receptor agonists of different therapeutic utility.

Note added in proof

A recent report (Eghbali, M., Curmi, J. P., Birnir, B. & Gage, P. W., *Nature* **388**, 71–75 (1997)) has demonstrated that the BDZ diazepam can increase the single-channel conductance of $GABA_A$ receptors in hippocampal neurones. If a proportion of the $GABA_A$ receptors present at the inhibitory synapses of granule cells behaved in this fashion, this novel BDZ action could be responsible for the small BDZ-induced enhancement of mIPSC amplitude shown in Fig. 1.

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