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BARBITURATE REGULATION OF KINETIC PROPERTIES OF THE $GABA_A$ RECEPTOR CHANNEL OF MOUSE SPINAL NEURONES IN CULTURE

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SUMMARY

1. Barbiturate regulation of the kinetic properties of γ-aminobutyric $acid_A$ (GABA) receptor channel chloride currents from somata of mouse spinal cord neurones were investigated using whole-cell and excised outside-out patch-clamp recording techniques.

2. GABA (2 μM), GABA (2 μM) plus phenobarbitone (PhB) (500 μM) and GABA (2 μM) plus pentobarbitone (PB) (50 μM), applied by pressure ejection from blunt perfusion micropipettes, evoked inward chloride currents when neurones or patches were voltage clamped at -75 mV and the chloride equilibrium potential was 0 mV. GABA receptor channel currents were increased by PhB and PB.

3. Single GABA receptor channel currents were recorded with a main conductance state of 27 pS and a less frequent subconductance state of 16·5 pS. The conductances of the two states were unchanged by the barbiturates.

4. The main conductance state kinetics were analysed. GABA alone or with the barbiturates gated the channel open singly and in groups of openings.

5. The barbiturates increased GABA receptor channel mean open time and shifted frequency histograms of channel open times to longer times.

6. Three exponential functions were required to fit the frequency histograms of GABA receptor channel open times, suggesting that the channel has at least three open states (O_1, O_2, O_3). The time constants for the exponential functions (0·9, 2·7 and 7·8 ms, respectively) were unchanged by the barbiturates. The increases in mean open times and the shifts of the open-time frequency histograms by the barbiturates were due to a reduction in relative frequency of occurrence of the two short open states (O_1 and O_2) and to an increase in the relative frequency of occurrence of the longest open state (O_3).

7. Frequency histograms of GABA receptor channel closed times were fitted with five exponential functions, suggesting that the channel has multiple closed states. None of the time constants nor areas of the exponential functions were significantly changed by the barbiturates.

8. For analysis, a burst was defined as openings surrounded by closures greater than a critical closed time, t_c, of 5 ms. For GABA (2 μM), frequency histograms of GABA receptor channel bursts were fitted with three exponential functions,

suggesting that the channel has three burst states (B_1, B_2, B_3). The B_1 burst state was probably a single opening to the O_1 open state while the B_2 and B_3 burst states were probably composed of multiple openings to the O_2 and O_3 open states. With both barbiturates, the relative frequency of occurrence of the two shortest burst states (B_1 and B_2) was reduced and that of the longest burst state (B_3) was increased. The time constant of the B_3 burst state was increased slightly by the barbiturates.

9. We suggest that barbiturates increase GABA receptor channel current by increasing the relative frequency of occurrence of the long (B_3) bursts which contain multiple long openings and decreasing the relative proportion of shorter (B_1 and B_2) bursts which contain shorter openings. This results in an increase in GABA receptor channel apparent mean open time and mean burst duration. Barbiturates do not measurably alter the closing rates of the open states, and therefore, do not alter their mean open times. Rather, barbiturates alter the rate constants which regulate entry into the three open states, such that entry into the longest, stable open state (O_3) is favoured over the two shorter and less stable open states (O_1 and O_2).

INTRODUCTION

The postsynaptic γ-aminobutyric $acid_A$ (GABA) receptor channel is composed of an oligomeric complex of polypeptide subunits which form a chloride channel associated with binding sites for GABA, benzodiazepines and barbiturates (Olsen, 1982; Schofield, Darlison, Fujita, Burt, Stephenson, Rodriguez, Rhee, Ramachandran, Reale, Glencorse, Seeburg & Barnard, 1987). GABA binds to the receptor and gates open the chloride channel (Curtis, Hösli, Johnston & Johnston, 1968; Hamill, Bormann & Sakmann, 1983). Barbiturates potentiate the inhibitory effects of GABA in the vertebrate central nervous system (Schmidt, 1963; Nicoll, 1972; Ransom & Barker, 1976; Macdonald & Barker, 1979). Based on studies using the noise analysis technique, it was proposed that: (1) the GABA receptor channel opens into a single open state with exponentially distributed open durations and (2) barbiturates potentiate GABAergic inhibition by increasing the mean duration of the single open state without altering the frequency of channel opening or the channel conductance (Barker & McBurney, 1979; Study & Barker, 1981; Barker, McBurney & MacDonald, 1982). However, the resolution of the noise analysis technique is low. Using the single-channel recording technique which has a higher temporal resolution (Hamill, Marty, Neher, Sakmann & Sigworth, 1981), single GABA receptor channel currents have been recorded and shown to be more complex (Jackson, Lecar, Mathers & Barker, 1982; Hamill *et al.* 1983; Sakmann, Hamill & Bormann, 1983; Bormann & Clapham, 1985; Martin, 1985; Mathers, 1985*a*; Bormann, Hamill & Sakmann, 1987; Macdonald, Rogers & Twyman, 1989). The GABA receptor channel has been shown to have multiple open and closed states (Macdonald *et al.* 1989) and to open in bursts (Hamill *et al.* 1983). The effect of barbiturates on the kinetic properties of GABA receptor channels has not been determined. We have used whole-cell and outside-out patch-clamp recording techniques to study the regulation of GABA receptor channel currents by the

barbiturates phenobarbitone (PhB) and pentobarbitone (PB), using mouse spinal cord neurones grown in cell culture.

METHODS

Cell culture

A single-cell suspension of neurones from mechanically dissociated spinal cords, dissected from 10 to 12-day-old murine fetuses, was grown in culture medium as previously described (Ransom, Neale, Henkart, Bullock & Nelson, 1977; Macdonald *et al.* 1989). The mothers were killed by cervical dislocation under CO_2 narcosis prior to removal of the fetuses. Cultures were maintained for 2–5 weeks prior to being used in these experiments.

Bathing and recording solutions

The same extracellular and intrapipette solutions consisting of a symmetric chloride salt solution were used for both whole-cell and excised outside-out recordings. The extracellular solution consisted of (in mM): 142 NaCl, 8·1 KCl, 1 $CaCl_2$, 6 $MgCl_2$, 10 glucose, 10 Na^+–HEPES (pH 7·4). The intrapipette solution consisted of (in mM): 153 KCl, 1 $MgCl_2$, 10 Na^+–HEPES, 5 EGTA, 1 NaOH, 2 KOH (pH 7·4). The specific glycine receptor antagonist strychnine (200 nM) (Sigma Chemical Co., St Louis, MO, USA) was added to all solutions to ensure that no glycine receptor channel currents were included in the analyses. Recordings were performed at room temperature (20–23 °C). This combination of extracellular and intrapipette solutions resulted in a chloride equilibrium potential (E_{Cl}) of 0 mV and a potassium equilibrium potential (E_K) of −75 mV.

Micropipettes

Patch recording micropipettes were fabricated up to 24 h prior to use. Drugs and GABA were applied from pressure ejection or diffusion micropipettes. To avoid dilution in the drug micropipette tips, the drug micropipettes were removed from the bathing solution whenever the drugs were not being applied. Prior to application, a pressure pulse (5–10 s) was applied distant from the recording site to eject diluted solution from the micropipette tip.

Equipment

Both whole-cell and patch-clamp recordings were performed using a Model L/M EPC-7 amplifier (LIST-Medical Instruments, Darmstadt, FRG). For whole-cell recordings, responses were low-pass filtered (3 dB at 600 Hz, 8-pole Butterworth) (A.P. Circuit Corporation. New York, NY, USA) and recorded on a Gould chart recorder (Gould Inc., Cleveland, OH, USA). Patch-clamp command potentials and single-channel currents were recorded on a video cassette recording system (VCR) (SONY SL-2700, modified to 0–20 kHz) via a digital audio processor (SONY PCM-501 ES, 14-bit, 44 kHz). Simultaneously, these data were recorded on a chart recorder (Gould Inc.) using a low-pass (3 dB at 1 kHz) 8-pole Bessel filter (Frequency Devices, Haverhill, MA, USA). For single-channel analysis the data were played back from the VCR system and digitized (8 kHz, 14 bit, Tecmar A/D converter, Cleveland, OH, USA) for computer (80386 based processors) analysis with a low-pass (3 dB at 1 kHz), 8-pole Bessel filter interposed. Digitized data segments ranged in length from 20–25 s. All recordings were performed at room temperature (20–23 °C).

Drug application

GABA, PhB and PB were obtained from Sigma Chemical Co. GABA (2 μM) was prepared as previously described and applied using pressure micropipettes (Nowak, Young & Macdonald, 1982). The barbiturates PhB and PB were dissolved in solution and serial dilutions provided a final concentration of 500 μM for PhB and 50 μM for PB. These concentrations have been shown previously to enhance GABA responses on mouse spinal cord neurones in cell culture without directly activating GABA receptor channel current (Macdonald & Barker, 1978; Schulz & Macdonald, 1981). A mixture of GABA (2 μM) and barbiturate was applied to the neurone or patch

membrane via pressure ejection micropipettes in the presence of a diffusion pipette containing the barbiturate. Micropipettes were moved to within 50 μm of the neurone or membrane only during the time of each application.

Whole-cell and outside-out patch recording

Whole-cell and excised outside-out patch recording techniques were similar to those of Hamill *et al.* (1981). The whole-cell recording technique was used to establish concentrations of PhB and PB that produced approximately equal peak current enhancement of the GABA-evoked whole-cell response. The recording intrapipette potential for both whole-cell and outside-out patch recording was voltage clamped at -75 mV (E_K).

Analysis of data

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.* 1989). GABA has been shown to activate chloride channel openings to at least four conductance states (Bormann *et al.* 1987). In the present study, only the more frequent or main conductance state (approximately 27–30 pS) was analysed. Detection of main conductance-state amplitude and duration were as previously described (Macdonald *et al.* 1989). Main conductance state open and closed durations were pooled and collated into frequency histograms using methods previously described (Macdonald *et al.* 1989).

Only patches with infrequent multiple channel activity and reproducible activity were used for analysis. Occasionally, after repeated GABA application, current activity decreased significantly or stopped for prolonged periods. Therefore, patches were accepted only if they were stable during the first two or three applications of GABA. Kinetic stationariness was assessed by analysing consecutive epochs during the 20 s drug application. The first 2 s were found to be non-stationary, probably due to diffusion time from the pressure ejection pipette. Records analysed 2 s after the first opening revealed stable open- and burst-duration kinetic properties.

Detected openings and closings less than twice the system dead time (dead time = 130 μs) including the low-pass 8-pole Bessel filter (3 dB cut-off at 1 kHz) were counted as unresolved openings or closings, respectively (McManus, Blatz & Magleby, 1987). Starting from at least twice the system dead time, histograms were fitted to a sum of exponentials using a locally modified non-linear curve-fitting routine (NFITS, M. Sloderbeck & C. J. Lingle, Florida State University, Tallahassee, FL, USA). Estimates of exponential area and time constant were obtained using the method of maximum likelihood estimation. Likelihood intervals ($m = 2$) were calculated for each parameter. The number of significant exponential functions necessary to fit the distributions was determined by fitting increasing numbers of functions until the χ^2 of the estimated fit and data was within the 95% confidence interval for accepting the null hypothesis (no difference between fit and data) (Colquhoun & Sigworth, 1983). Distributions of open and closed times were fitted using the same bin widths and over the same range for GABA alone and GABA with PhB or PB.

The mean open and closed times measured in this study were apparent or observed times. Due to the presence of missed or unresolved openings, the apparent open times are greater than the true times (Colquhoun & Sigworth, 1983). Correction of mean open time for missed openings can be obtained by re-estimating the mean open time from the exponential function fits of the open-time distributions. This type of correction, however, does not correct for the effect of missed closures. A similar correction can be made for mean closed time. Simultaneous correction for both missed openings and missed closures cannot be done without a specific model of the gating mechanism, which at present is unavailable (McManus *et al.* 1987).

Definition of bursts

A burst may be defined as an opening or 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 such 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 GABA (2 μM) (Colquhoun & Sakmann, 1985; Macdonald *et al.* 1989). This critical closed time would result in misclassification of 2·43% of the closures originating from each of the closed-time distributions.

RESULTS

Whole-cell currents

GABA (2 μM) evoked an inward current and associated increase in membrane conductance (Fig. 1*A*1 and *B*1). In the presence of PhB (500 μM) (Fig. 1*A*2) or PB

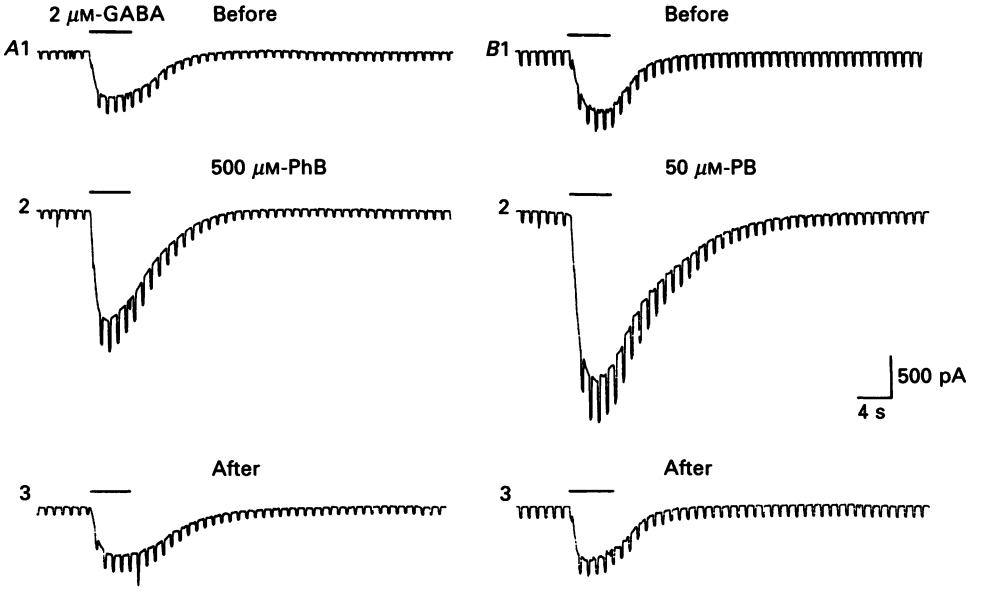

Fig. 1. GABA receptor channel current was enhanced by PhB and PB. Neurones were held at -75 mV (E_K) using the whole-cell patch-clamp technique. E_{Cl} was 0 mV. Small hyperpolarizing commands (200 ms) were applied at 1 Hz, and the small inward currents required for these commands are apparent in the current recordings. *A*, GABA (2 μM) applied for 5 s to the soma produced an inward current and associated increase in membrane conductance (*A*1) which was potentiated by PhB (500 μM) (*A*2). One minute after removal of PhB, the response returned to control (*A*3). *B*, in a different neurone, GABA (2 μM) applied for 5 s produced an inward current and associated increase in membrane conductance (*B*1) which was potentiated by PB (50 μM) (*B*2). The response returned to control 1 min after removal of PB (*B*3).

(50 μM) (Fig. 1*B*2), peak GABA receptor channel current and conductance responses were enhanced. At these concentrations, PhB produced an average increase in peak current of 113% (twenty-three trials, four cells) and PB produced an average increase of 122% (six trials, five cells). After removal of either barbiturate, GABA receptor channel currents returned to pre-drug levels within 1 min (Fig. 1*A*3 and *B*3). These concentrations of PhB and PB were used in the subsequent excised outside-out patch clamp recordings of single GABA receptor channel currents. Direct application of barbiturate at these concentrations did not evoke current (not illustrated).

Single-channel currents

Excised outside-out patches were obtained at 0 mV (E_{Cl}) and abundant outward potassium currents were commonly recorded. Following patch hyperpolarization to

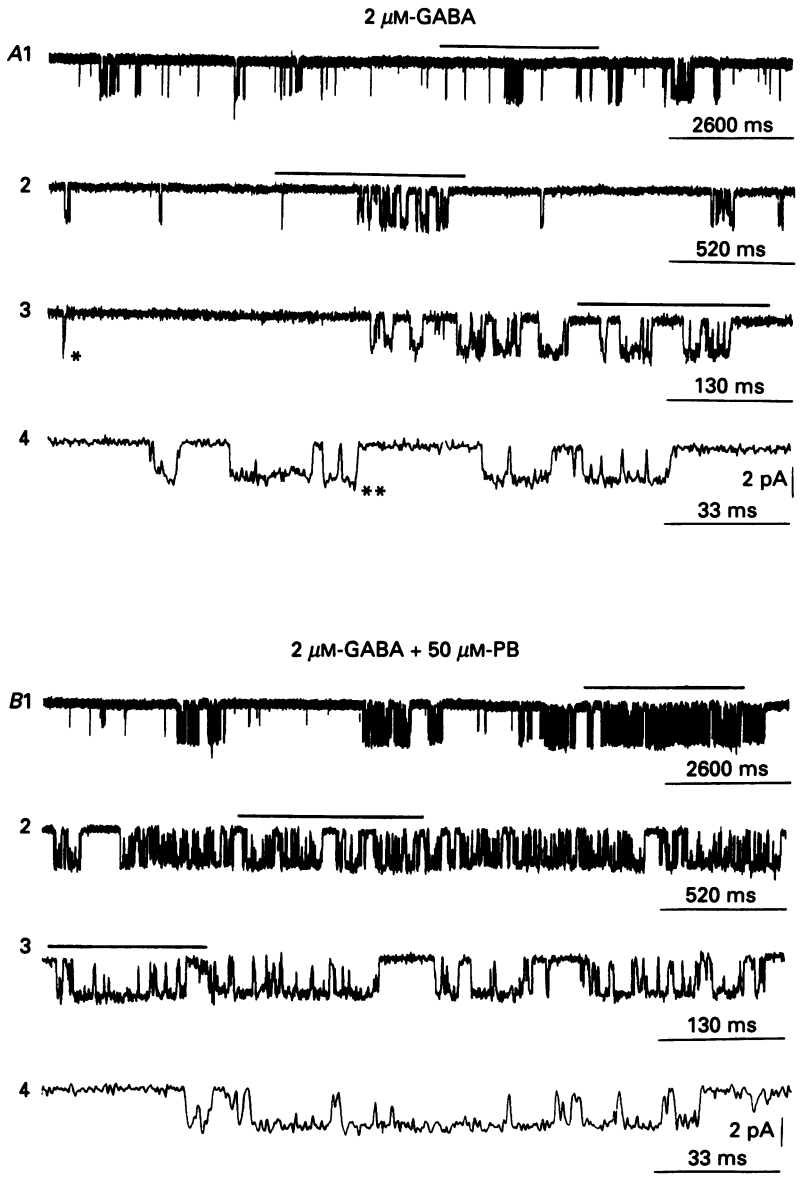

Fig. 2. *A*, GABA (2 μM) evoked bursting single-channel currents in outside-out patches from spinal cord neurones held at −75 mV. Solutions were as described in the text. Inward unitary currents are shown at increasing time resolution (*A*1–4). The portion of current record under the horizontal line above each current trace is presented expanded in time in the tracing below it. GABA receptor channel currents consisted of brief, single (*) and longer, more complex (**) groups of currents. Time calibration for each trace is shown on the right below the trace. Current calibration of lower right applies throughout. *B*, GABA (2 μM) evoked prolonged bursting currents in PB (50 μM) from an outside-out patch held at −75 mV. Inward currents are shown at increasing time resolution (*B*1–4). The portion of current record under the horizontal line above each current trace is presented expanded in time in the tracing below it. Time calibration for each trace is shown on the right below the trace. Current calibration at lower right applies throughout.

-75 mV (E_K), the potassium currents became inapparent. At -75 mV, rare spontaneous currents consisting primarily of very brief openings were recorded (not illustrated). Following application of GABA (Fig. 2*A*), bursting inward currents were observed in 80% of patches. Only patches with reproducible activity and rare

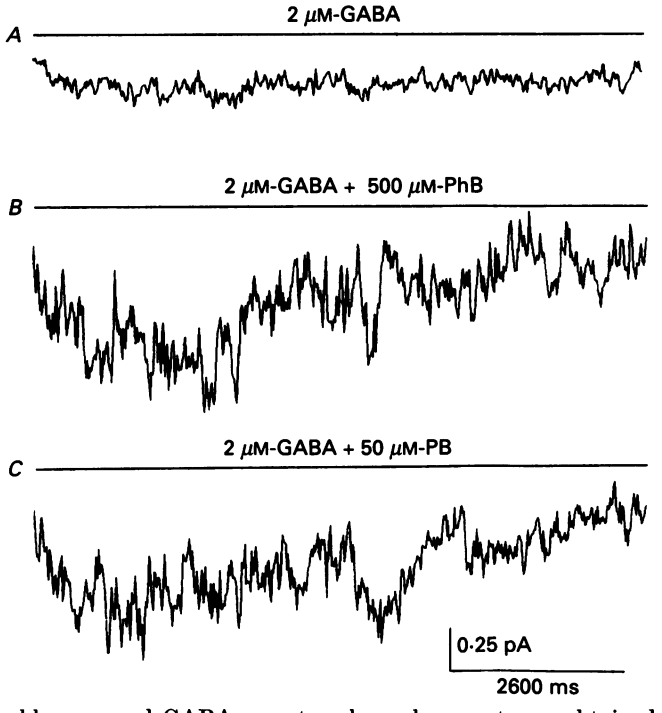

Fig. 3. Ensemble-averaged GABA receptor channel current was obtained by summing recordings of currents from several outside-out patches evoked by 20 s applications of GABA (2 μM) (*A*), GABA (2 μM) plus PhB (500 μM) (*B*) and GABA (2 μM) plus PB (50 μM) (*C*). Inward current was down-going. Calibration at lower right applies throughout.

multiple channel currents were used for kinetic analysis. GABA receptor channel currents could be blocked by bicuculline (10 μM) or picrotoxin (50 μM) (not illustrated). At increasing time resolution (Fig. 2*A*1–4), openings evoked by GABA could be resolved into openings that appeared to be brief and occur in isolation (Fig. 2*A*3,*) and into groups of openings interrupted by brief closures (Fig. 2*A*4,**). In the presence of PB (Fig. 2*B*), GABA receptor channel current was increased. At increasing time resolution, it was apparent that the barbiturates prolonged channel openings and increased the number of openings occurring close together (Fig. 2*B*1–4). GABA receptor channel currents in the presence of PhB were similar to those in PB (not illustrated).

It was difficult to be certain that any patch contained only a single GABA receptor channel. Patches were selected to contain rare multiple simultaneous channel openings. In all patches analysed, multiple simultaneous openings constituted 1·35% of all detected openings.

Multiple recordings of currents evoked by 20 s applications of GABA or GABA

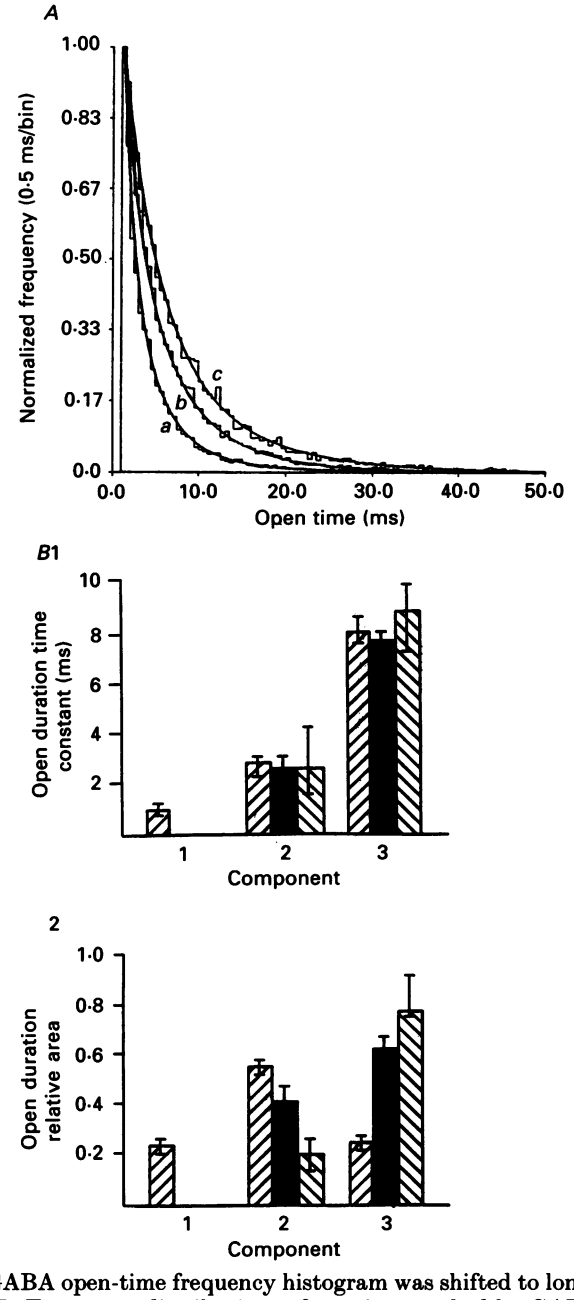

Fig. 4. *A*, the GABA open-time frequency histogram was shifted to longer open durations by PhB and PB. Frequency distributions of openings evoked by GABA (2 μM, *a*) and in PhB (500 μM, *b*) or PB (50 μM, *c*) were put into 0·5 ms bins and displayed over a range of 1–50 ms. Distributions were normalized and overlaid to display relative frequency distributions. Histograms were fitted with three exponential functions for GABA alone and two exponential functions for GABA plus barbiturate. Curves were drawn according to the fits (see text). *B*, the relative areas (*B*2), but not the time constants (*B*1), of the exponential components of the open-time frequency distributions were altered by the barbiturates. Histograms were best fitted with three exponential functions for GABA alone and two exponential functions for GABA plus PhB and GABA plus PB. For GABA

plus barbiturate were summed and averaged to evaluate the average single-channel current evoked per GABA application. For these averaged currents, simultaneous multiple channel openings were not rejected. GABA (115 applications) produced an average current of 0·33 pA (Fig. 3*A*). GABA applied with PhB (twenty-seven applications, Fig. 3*B*) or PB (thirty-three applications, Fig. 3*C*) both produced average currents of 0·49 nA (48% increase in mean current). The time from the start of GABA application to peak current amplitude was similar to that of whole-cell responses (Fig. 1) and was probably due to the diffusion time from the pressure pipette. In the presence of the barbiturates, average current declined slightly over time, possibly due to desensitization.

Openings with at least two different current amplitudes were commonly recorded, representing two different conductance states. The larger state (27·0 ± 3·4 pS chord conductance; mean ± S.D.) occurred more frequently and was used in this analysis. Conductance of the main channel evoked by GABA in the presence of PhB (27·0 ± 1·7 pS) or PB (27·4 ± 1·7 pS) was unchanged. The relative proportion of the total current attributed to the subconductance state (16·5 ± 0·22 pS; mean ± S.E.M.) was unchanged by the barbiturates (10·2 ± 1·5%). Since barbiturates enhanced GABA receptor channel current without altering channel conductance, the temporal characteristics of opening and closing of the channel were analysed.

Open states were increased by the barbiturates

GABA evoked 14·2 openings per second (22249 openings). In the presence of PhB or PB, GABA evoked 12·8 (21158 openings) and 13·6 (23802 openings) openings per second, respectively. Mean corrected (see Methods) open time for GABA (2 μM) was 3·5 ms. In PhB, the mean corrected open time for GABA was increased to 5·4 ms, and in PB, it was increased to 8·3 ms. When applied alone, GABA gated the channel open 5·6% of the time. In PhB or PB, GABA gated the channel open 7·5 and 12·5% of the time, respectively. Thus, both barbiturates altered the temporal characteristics of channel opening.

To determine the basis for the barbiturate increase of mean open time for GABA receptor channel currents, open durations were collated into frequency histograms and normalized (Fig. 4*A*). Both PhB and PB shifted the distributions of the openings to longer open times. The frequency histograms of GABA receptor channel openings were best fitted to a sum of three exponential functions. The exponential component with the shortest time constant was designated component 1, that with the second longest component 2 and that with the longest component 3. In contrast, the

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alone, components 1, 2 and 3 correspond to exponential functions with the shortest, intermediate and longest time constants, respectively. Error bars represent likelihood intervals ($m = 2$). The shorter and longer time constants resolved in GABA plus PhB and GABA plus PB distributions overlapped the GABA components 2 and 3 respectively (*B*1). A time constant corresponding to component 1 could not be resolved. The relative area of component 3 was significantly increased by the barbiturates (*B*2). The relative area of component 2 was decreased. ▨, 2 μM-GABA; ■, 2 μM-GABA + 500 μM-PhB; ▧, 2 μM-GABA + 50 μM-PB.

frequency histograms for GABA receptor channel openings in PhB and PB were fitted best with only two exponential functions.

The time constant of component 1 for GABA was 0·9 ms (Fig. 4*B*1). In the barbiturates, component 1 time constants were not resolved. The time constants of components 2 and 3 for GABA were 2·7 and 7·8 ms, respectively. With the barbiturates, the time constants for components 2 and 3 (2·6 and 7·4 ms for PhB and 2·5 and 8·7 ms for PB, respectively) were not significantly different from those for GABA alone (Fig. 4*B*1). Thus, the barbiturates did not increase mean open time by changing the individual component time constants.

Since component 1 could not be resolved in the open-time histograms following barbiturate application, it is likely that the increase in mean open time was due to an increase in the relative frequency of occurrence of longer openings. The relative area of each component was a measure of the relative frequency of occurrence of openings contributed by each component. For GABA, the relative area of component 1 was 0·23 (Fig. 4*B*2). In PhB and PB, no component 1 time constants were resolved. For GABA, the relative area of component 2 was 0·53. In the presence of PhB and PB, the relative areas of component 2 decreased to 0·41 and 0·20, respectively (Fig. 4*B*2). For GABA, the relative area of component 3 was 0·24. In PhB and PB, the relative areas of component 3 increased to 0·59 and 0·80, respectively (Fig. 4*B*2). Thus, the barbiturates increased GABA receptor channel mean open time by increasing the relative frequency of occurrence of component 3 openings.

Closed states were unaltered by the barbiturates

The distributions of closed times ranged from the shortest accurately detected duration of 260 μs to more than 2 s. Openings to the lower conductance state or multiple simultaneous openings were eliminated from the analysis. Similar to the open times, closed times were collated into normalized frequency histograms. Due to the wide range of closed times, closed-time frequency histograms were obtained using two different bin magnitudes. For curve fitting, the short closed-time histogram ranged from 0·50–150·0 ms in bin widths of 0·5 ms (Fig. 5*A*). The long closed-time histogram ranged from 12·5–3750 ms in bind widths of 12·5 ms (Fig. 5*B*). Ranges of closed times were altered for display of the details of the closed-time histograms (Fig. 5). Visual inspection of the closed-time histograms revealed minimal alteration by the barbiturates. Three exponential functions were required to fit each closed-time histogram (Fig. 5). When compared among the histograms, five exponential components could be resolved for GABA. None of the closed time constants or the relative areas of the exponential functions were significantly altered by PhB or PB. The average time constants were $0{\cdot}21 \pm 0{\cdot}01$, $1{\cdot}9 \pm 0{\cdot}1$, $17{\cdot}8 \pm 0{\cdot}8$, $109{\cdot}4 \pm 7{\cdot}4$ and 520 ± 45 ms (mean $\pm$ S.E.M.).

Burst durations were increased by the barbiturates

Single-channel openings may occur in groups or bursts. Bursts were defined as one or more openings separated by closings greater than a critical time, t_c. We have shown previously that since the closed time constants of components 1 and 2 were unaffected by GABA concentration, a value for t_c (5 ms) was calculated between the closed time components 2 and 3 (see Methods) (Macdonald *et al.* 1989). Since the

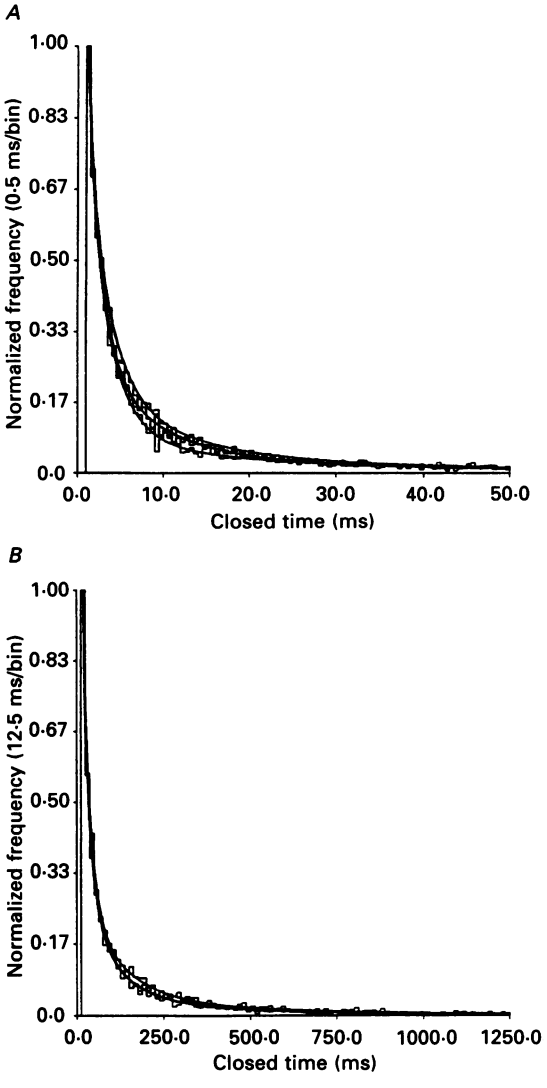


Fig. 5. Closed-time frequency histograms over two different time ranges were fitted with multiple exponential functions and were unaltered by the barbiturates (see text). Distributions from GABA (2 μM, bottom curve in *A*) alone, GABA (2 μM) plus PhB (500 μM, middle curve in *A*) and GABA (2 μM) plus PB (50 μM, top curve in *A*) were normalized and overlaid. *A*, short closed times were put into 0·5 ms bins and displayed over a range of 1–50 ms. *B*, long closed times were put into 12·5 ms bins and displayed over a range of 25–1250 ms. The short and long closed-time frequency histograms were each fitted with three exponential functions.

barbiturates did not alter the closed time constants, a t_c of 5 ms was used for the burst analyses in this study.

Barbiturates did not alter or slightly reduced GABA receptor channel burst frequency (Table 1). Mean durations of bursts, however, were increased by the barbiturates. GABA evoked bursts with an overall mean corrected (see Methods)

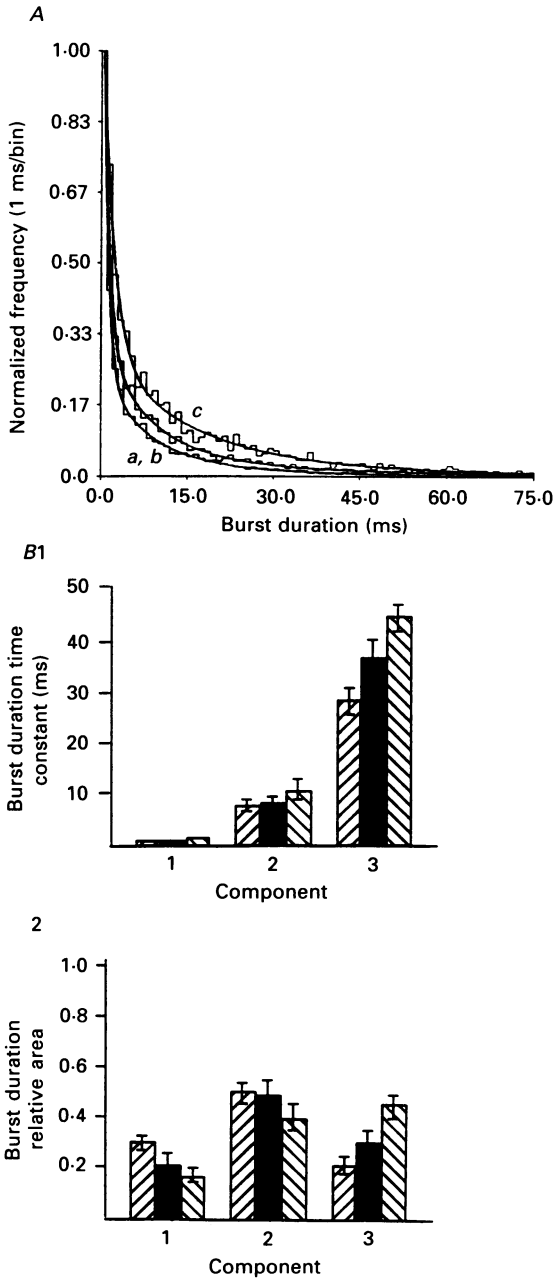
TABLE 1. Burst properties

	2 μM-GABA	+500 μM-PhB	+50 μM-PB
Bursts per second	6·7	5·6	5·4
Mean corrected burst duration (ms)	10·0	14·1	21·4
Percentage intraburst open time	82·4	87·7	91·2
Mean number of openings per burst	2·1	2·3	2·5
Mean intraburst closed time (ms)	1·6	1·5	1·4
Percentage in burst state	6·9	8·5	8·8
Number of bursts	10708	9154	9542

duration of 10·0 ms. In PhB, GABA receptor channel mean corrected burst duration increased to 14·1 ms, and in PB it increased to 21·4 ms. The mean number of openings per burst was increased slightly from 2·1 to 2·3 by PhB and to 2·5 by PB. In the presence of the barbiturates, the increase in GABA receptor channel burst duration resulted in an increase in the percent of time spent in a burst from 6·9%, to 8·5 and 8·8% in PhB or PB, respectively. The percentage of open time within a burst (percentage intraburst open time) was increased slightly by PhB and PB (Table 1), and the mean intraburst closed time was decreased slightly by PhB and PB (Table 1).

Burst durations were collated into normalized frequency histograms (Fig. 6*A*). Both PhB and PB shifted the histograms of burst durations to longer durations, consistent with an increase in the relative frequency of occurrence of longer bursts. Pentobarbitone produced a greater shift to the right than PhB. The burst histograms were best fitted with three exponential functions, suggesting three burst components. Components 1 through 3 represented bursts with the shortest to the longest time constants. The barbiturates had little effect on the first two time constants of the three burst components. The time constant for component 1 was not significantly changed (mean $0{\cdot}99 \pm 0{\cdot}24$ ms) by the barbiturates (Fig. 6*B*1). The component 2 time constant was 7·8 ms in GABA. It was not changed by PhB (8·1 ms) but was slightly increased by PB (10·5 ms). The component 3 time constant was 27·5 ms in GABA. It was increased to 35·7 ms in PhB and to 43·1 ms in PB (Fig. 6*B*1). The barbiturates significantly altered the relative areas of the three burst components. The relative area of component 1 was 0·30 in GABA. It was decreased to 0·22 by PhB and to 0·17 by PB (Fig. 6*B*2). Burst component 2 relative area was 0·49 in GABA alone. It was not significantly changed (0·48) by PhB but was decreased to 0·39 by PB (Fig. 6*B*2).

Fig. 6. *A*, burst-duration frequency histograms were shifted to longer durations in the presence of the barbiturates. GABA (2 μM, *a*), GABA (2 μM) plus PhB (500 μM, *b*) and GABA (2 μM) plus PB (50 μM, *c*) burst durations were put into 0·5 ms bins and displayed over a range of 1–75 ms. The histograms were each fitted with three exponential functions. *B*, the time constants (*B*1) and relative areas (*B*2) of exponential functions fitted to burst-duration frequency histograms were altered by the barbiturates. Curves were best fitted with three exponential functions for GABA, GABA plus PhB and GABA plus PB. Components 1, 2 and 3 corresponded to exponential functions with the shortest, intermediate and longest time constants, respectively. Error bars represent likelihood



intervals ($m = 2$). Error ranges smaller than the line thickness were not shown. Time constants for components 1 and 2 overlapped (*B*1). Time constants for component 3 were shifted to longer times in GABA plus PhB and in GABA plus PB compared to GABA alone. Compared to GABA alone, the relative area of component 1 decreased in PhB and PB (*B*2). The relative area of component 2 was unchanged or slightly decreased, and the relative area of component 3 increased. ▨, 2 μM-GABA; ■, 2 μM-GABA + 500 μM-PhB; ▧, 2 μM-GABA + 50 μM-PB.

Burst component 3 relative area was increased by both barbiturates (Fig. 6*B*2). In GABA alone, it was 0·21. It was increased to 0·30 by PhB and to 0·44 by PB. Thus, both barbiturates increased the relative frequency of occurrence of the longest burst component and decreased the relative frequency of occurrence of the two shorter burst components.

DISCUSSION

Comparison to other studies

Using outside-out patches obtained from mouse spinal cord neurones grown in cell culture, we investigated PhB and PB modulation of the main conductance level of single GABA receptor channel currents. As previously reported, we recorded a main conductance state of 27 pS in symmetrical chloride solutions at −75 mV (E_K) (Macdonald *et al.* 1989). The conductance of the GABA receptor channel main state was not altered by PhB or PB. This was consistent with results obtained using fluctuation noise analysis (Barker & McBurney, 1979; Study & Barker, 1981). Thus, enhancement of GABA receptor channel current by barbiturates was due to an increase in the frequency of occurrence of channel opening and/or a change in the gating properties of the channel.

Phenobarbitone applied iontophoretically at an unknown concentration was reported to increase 'mean channel lifetime' of GABA channel openings from 24 to 120 ms (Barker & McBurney, 1979). In similar studies, PB (50 μM) also increased 'mean channel lifetime' of GABA channel openings from 21 to 73 ms (Study & Barker, 1981). The percentage increase by PB of 'mean channel lifetime' was greater than the percentage increase in total current, and thus, PB was felt to decrease channel opening frequency. Due to limited resolution in these studies (approximately 100–200 Hz), it was unlikely that single openings of channels were detected. Rather, it was more likely that bursts of openings were resolved and that 'mean channel lifetime' was an average measure of longer burst durations. Consistent with this, we report that PhB and PB increased overall mean burst durations 55 and 144%, respectively. Also, PhB and PB decreased burst frequency 16 and 19%, respectively. Thus, conclusions derived from earlier low resolution fluctuation analysis can be reinterpreted. The barbiturates PhB and PB increased GABA receptor channel burst duration and decreased burst frequency.

Pentobarbitone enhancement of GABA receptor channel currents has also been studied using single-channel recording techniques (Mathers, 1985*b*). Pentobarbitone (200 μM) was reported to 'increase the tendency for GABA (1 μM) to evoke bursts of openings'. In our study, however, burst frequency was not increased. Rather, longer bursts were evoked. Thus, in the presence of the barbiturates, GABA promoted longer bursts rather than increasing their frequency.

Open properties

In the present study, mean open time of GABA receptor channels was increased by PhB and PB, but channel opening frequency was unaffected. Three exponential functions were required to fit open-time frequency histograms, suggesting that the GABA receptor channel opened into at least three open states (O_1, O_2 and O_3).

Following addition of PhB or PB, only two open time constants were obtained. A component that may correspond to state O_1 in GABA could not be reliably estimated in GABA with PhB or PB. The two open time constants resolved in PhB and PB were not significantly different from the time constants for the two long open states (O_2 and O_3) obtained with GABA alone. Thus, the barbiturates did not alter the mean open times of the resolvable open states. Rather, the barbiturates increased the probability of opening into the longer, more stable open state (O_3), and reduced the probability of opening into the shortest open states (O_1 and O_2). These results suggest that the barbiturates did not alter the probability of opening or closing of GABA receptor channels, but rather, they increased the relative probability of opening into stable, long open states rather than unstable, short open states.

The enhancement of GABA receptor channel current by the barbiturates was different than the enhancement of current by increasing GABA concentration. At low GABA concentrations (0·5–5 μM), we have previously reported the following open properties of GABA receptor channel currents recorded in outside-out patches obtained from mouse spinal cord neurones in culture (Macdonald *et al.* 1989): (1) increased current with increased GABA concentration resulted from an increase in channel opening frequency and mean open duration; (2) mean channel open time increased with GABA concentration due to a shift in the proportion of openings from the short-lived state (O_1) to the more stable long states (O_2 and O_3). We suggested that O_1 openings were from the singly liganded receptor while the O_2 and O_3 openings were from the doubly liganded state. In contrast to the results found with increasing GABA concentration, the frequency of GABA receptor channel openings was not increased by the barbiturates, and the barbiturates increased the relative occurrence of the O_3 state relative to both the O_1 and O_2 states.

Closed properties

The absence of a barbiturate effect on closed time constants or on the relative frequency of occurrence of the closed components suggests: (1) that barbiturates did not affect GABA receptor channel opening rates or closed state-to-closed state transition rates or (2) that barbiturates altered rate constants between specific closed states or opening rate constants, but alteration of these rate constants did not result in a detectable change in the mean dwell time of the closed states, possibly due to the presence of other exit rate constants that were unaffected by the barbiturates. Furthermore, since the barbiturates did not alter the closed time constants, the critical closed time constant, t_c, determined for GABA receptor channel bursts should be the same for bursts in the presence of the barbiturates.

Burst properties

Mean durations of GABA receptor channel bursts were increased by PhB and PB, but burst frequencies were reduced. Frequency histograms for GABA receptor channel burst durations with and without barbiturates were fitted with three exponential functions, suggesting three burst states (B_1, B_2 and B_3). Since the B_1 state time constant was similar to the O_1 state time constant for GABA, this suggested that the B_1 state was produced by single openings to the O_1 state. In the presence of the barbiturates, however, no short time constant (O_1 state) was resolved

from the open-time frequency histogram. However, the time constants for the B_1 state in the presence of the barbiturates was similar to the time constants for the O_1 open state and B_1 burst state for GABA. These results suggest that the O_1 state occurs in the presence of the barbiturates, but at a very low frequency. The O_1–B_1 state can be resolved in the burst frequency histogram because openings in B_1 state bursts were primarily single O_1 state openings while the openings in B_2 and B_3 state bursts were composed primarily of multiple O_2 and O_3 state openings. Thus, the relative frequency of the B_1 state bursts was higher than the relative frequency of O_1 state openings.

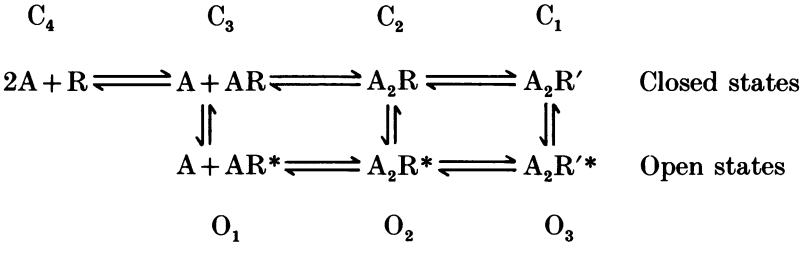
B_2 and B_3 state bursts were probably composed of two or more openings to the O_2 and O_3 states since the burst duration time constants were much longer than the time constants for the O_2 and O_3 states. Since the barbiturates did not alter the closing rates of the O_2 and O_3 states, it is likely that in addition to an increase in the relative frequency of O_3 state openings, there was an increase in the number of openings per burst to account for the increase in B_3 state burst duration. To determine this, an analysis of intraburst kinetics is required which is beyond the scope of the present paper.

The effect of the barbiturates on GABA receptor channel bursts was different than the effect of increasing GABA concentration. At low GABA concentrations (0·5–5 μM), burst frequency and mean burst duration increased with concentration (Macdonald *et al.* 1989). In contrast, barbiturates increased GABA receptor channel current and decreased burst frequency. The current enhancement was due solely to burst prolongation. Also, there was no change (PhB) or a slight relative reduction (PB) in B_2 state burst frequency rather than an increase. Thus, barbiturates increased the relative frequency of B_3 state bursts while increasing GABA concentration increased the relative frequency of both B_2 and B_3 state bursts.

Site of barbiturate regulation of GABA receptor channel gating

In a preliminary model of GABA receptor channel gating, we proposed that the GABA receptor channel has at least three open and four closed states (Macdonald *et al.* 1989). In the model (see below), A represents an agonist molecule, R represents the closed receptor channel, R* represents the open receptor channel and R′ represents an additional conformation of the doubly liganded receptor channel. Closed states C_1–C_4 represent bound and unbound closed receptor states. The open states O_1, O_2 and O_3 correspond to the open conformations AR*, A_2R* and A_2R'*.

We proposed that the O_2 and O_3 states arose after the receptor was doubly liganded by GABA. The O_1 state largely occurred when the receptor was singly liganded. In the present study, barbiturates did not alter apparent open-state closing



rates (O_2 and O_3 time constants were unchanged) and did not alter apparent opening rates (no change in the closed time constants or areas). The barbiturates only increased the probability of occurrence of the O_3 open state relative to the O_1 and O_2 open states. We suggest that the barbiturates bind to a regulatory site on GABA receptors which alters the distribution among open states by altering those rate constants which regulate entry into the open states (see above). Barbiturates enhance entry into the stable, longest open state (O_3) relative to the less stable, shorter states (O_1 and O_2). This effect of the barbiturates would account for several observations. The O_1 state would rarely occur since its probability of opening would be low relative to the other two states. Similarly the O_2 state would be observed less frequently since its probability of opening would be less than that of the O_3 state. As a result, the O_3 state would be observed relatively more frequently. The closing rates of the open states would be unaffected. In this model, however, the closed-state time constants would be altered for those states connected to the three open states. Such an alteration in time constants was not observed, suggesting that our model is incomplete or that the changes in rate constants produced by the barbiturates did not result in a detectable alteration in mean dwell times of the closed states. These conclusions must be considered preliminary since a unique kinetic model involving seven states is complex and cannot be proposed at the present time. Models containing multiple open and closed states are equally well described by multiple Markovian kinetic schemes (Blatz & Magleby, 1986), making it difficult to determine a unique kinetic scheme for GABA receptor channel gating and a specific site of action for the barbiturates. While we are unable to propose a unique model for GABA receptor channel gating and for the site of action of the barbiturates, the observations presented in this paper provide constraints that can be applied in the development of more specific models of GABA receptor channel gating.

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