

NEUROSTEROID REGULATION OF GABA_A RECEPTOR SINGLE-CHANNEL KINETIC PROPERTIES OF MOUSE SPINAL CORD NEURONS IN CULTURE

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SUMMARY

1. Single-channel kinetics of steroid enhancement of single γ -aminobutyric acid_A (GABA) receptor currents obtained from somata of mouse spinal cord neurones in culture were investigated using the excised outside-out patch-clamp recording technique. GABA ($2\ \mu\text{M}$) and GABA ($2\ \mu\text{M}$) plus androsterone (5α -androstan- 3α -ol-17-one, AND, $10\ \text{nM}$ – $10\ \mu\text{M}$) or pregnanolone (5β -pregnan- 3α -ol-20-one, PRE, $100\ \text{nM}$ – $10\ \mu\text{M}$) applied by pressure ejection from micropipettes evoked inward currents when patches were voltage clamped at $-75\ \text{mV}$ in symmetrical chloride solutions. Averaged GABA receptor currents were increased in the presence of the steroids.

2. GABA receptor currents were recorded with at least two conductance levels, a predominant or main-conductance level of about $28\ \text{pS}$ (which contributed 96% of the current evoked) and a minor or sub-conductance level of about $20\ \text{pS}$. The current amplitudes of the two conductance levels were unchanged by the steroids. The gating (opening and closing) kinetics of both of the conductance levels were analysed. Findings for the main-conductance level are summarized below.

3. Both steroids increased the average GABA receptor channel open duration. Consistent with the increased GABA receptor channel average open duration, the steroids shifted frequency histograms of GABA receptor channel open durations to longer durations. Three exponential functions were required to fit best the frequency histograms of GABA open durations, consistent with at least three kinetic open states of the main-conductance level. Time constants obtained from the GABA receptor channel open-duration frequency histograms were unchanged in the presence of the steroids. The basis for the increased average GABA receptor channel open durations by the steroids was due to an increased relative proportion of the two longer open-duration time constants. The GABA receptor channel average open durations were increased by AND and PRE in a concentration-dependent manner by shifting the proportion of openings to the longer open time constants. At a concentration of $10\ \mu\text{M}$, the prolongation of the average open duration was decreased, suggesting that the GABA receptor channel was blocked by these steroids.

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4. GABA receptor channel opening frequency was increased and average channel-closed duration was decreased by AND or PRE. Consistent with this, areas of the frequency histograms of channel closed durations were shifted to shorter durations. Closed frequency distributions were fitted best with five to six exponential functions, suggesting that the channel had multiple kinetic closed states. The three briefest time constants were not greatly altered by the steroids.

5. The average durations of GABA receptor channel bursts (groups of openings separated by closures greater than 5 ms) were increased by the steroids. The increased average burst durations were AND and PRE concentration-dependent, but time constants obtained from the GABA receptor channel burst-duration frequency histograms were unchanged in the presence of the steroids. The basis for the increased average burst durations was due to a shift in the proportion of bursts with shorter time constants to bursts with longer time constants. The steroids did not alter intrinsic burst properties of the GABA receptor channel, but rather, increased the likelihood of longer bursts which were comprised primarily of longer-duration openings.

6. The steroids also enhanced averaged currents resulting from the sub-conductance level. Qualitatively similar findings for the regulation of the open durations of the sub-conductance level were found.

7. These results suggest that AND and PRE enhanced GABA receptor current. The steroid enhancement of GABA receptor current was due to an increase in channel-opening frequency and an increase in the probability of opening of longer openings without altering the intrinsic dwell times or open time constants of the GABA receptor main-conductance channel. The mechanism for prolongation of average open and burst durations was similar to that described for barbiturates, but a significant change in channel-opening frequency has not been a prominent effect observed for barbiturates. Although it has been suggested that steroids and barbiturates bind to different sites and their differential effect on opening frequency seems to corroborate this, the mechanism for the observed prolongation of the GABA receptor main-conductance level was similar to that described for barbiturates. This suggests that these steroids and barbiturates may regulate the GABA receptor channel through at least one common effector mechanism.

INTRODUCTION

Although the effects of gonadal hormones on sexual characteristics are recognized, a number of endogenous and synthetic steroids and their derivatives have been shown to exert effects on the central nervous system which include sedation, hypnosis, anaesthesia and behavioural changes (McEwen & Parsons, 1982). The effects of these 'neurosteroids' can be induced relatively acutely, suggesting that their actions may be independent of the known intracellular actions of progestin steroids. Indeed, a variety of steroids and their derivatives have been demonstrated to interact with the γ -aminobutyric acid_A (GABA) receptor, and several neurosteroids have been shown to be potent stereoselective allosteric modulators of the GABA receptor (Harrison & Simmonds, 1984; Barker, Harrison, Lange & Owen, 1987; Callachan, Cottrell, Hather, Lambert, Nooney & Peters, 1987; Cottrell, Lambert & Peters, 1987; Harrison, Majewska, Harrington & Barker, 1987; Gee,

Bolger, Brinton, Coirini & McEwen, 1988). It has been proposed that some of the physiological and pathological effects of neurosteroids are mediated through the GABA receptor (Callachan *et al.* 1987; Mistry & Cottrell, 1990). Since these steroids can interact with GABA receptors at physiological concentrations, it has been speculated that physiological variability of these hormones or their metabolites contribute to behavioural changes and alter seizure susceptibility (Rosciszewska, Buntner, Guz & Zawisza, 1986; Majewska, Ford-Rice & Falkay, 1989). Neurosteroids also have been demonstrated to be synthesized and metabolized in the brain (Hu, Bourreau, Jung-Testas, Robel & Bailieu, 1987; Purdy, Morrow, Blinn & Paul, 1990).

The basis for neurosteroid interaction with the GABA receptor remains unclear. Progesterone metabolites and the synthetic anaesthetic steroid, alphaxalone, have been shown to modulate TBPS, GABA, muscimol, and benzodiazepine binding to the GABA receptor (Simmonds, Turner & Harrison, 1984; Majewska, Harrison, Schwartz, Barker & Paul, 1986; Harrison *et al.* 1987; Gee *et al.* 1988; Im, Blakeman, Davis & Ayer, 1990). Barbiturates also have been shown to modulate *S-t*-butylbicyclophosphorothionate (TBPS) and benzodiazepine binding suggesting that steroids and barbiturates have closely associated binding sites, but no common binding site for them has been demonstrated (Peters, Kirkness, Callachan, Lambert & Turner, 1988; Kirkness & Turner, 1988). Further supporting evidence for separate steroid and barbiturate binding sites has included observations that they differentially modulate binding of TBPS (Gee *et al.* 1988) and benzodiazepines (Harrison *et al.* 1987) and that they differentially reduced non-competitive antagonism of the GABA receptor channel by picrotoxin (Kirkness & Turner, 1988). Also, although high concentrations of both steroids and barbiturates have been shown to directly activate the GABA receptor, direct GABA receptor activation by high concentrations of steroids can be further enhanced by low concentrations of barbiturates (Callachan *et al.* 1987; Cottrell *et al.* 1987).

Structurally different steroids have been shown to either potentiate or antagonize GABA responses, and details of the channel kinetic mechanisms of action for the enhancement or reduction of GABA receptor function remain unknown (Callachan *et al.* 1987; Majewska, Mienville & Vicini, 1988). The reversal potential and conductance of the GABA receptor chloride selective channel were unaltered by steroids. Alphaxalone, androsterone (AND), pregnanolone (PRE), and other progesterone metabolites enhanced GABA receptor function and have been postulated to potentiate GABA responses in a 'barbiturate-like' fashion (Harrison & Simmonds, 1984; Majewska *et al.* 1986; Barker *et al.* 1987; Callachan *et al.* 1987; Cottrell *et al.* 1987; Harrison *et al.* 1987). Similar to pentobarbitone, prolongation of average channel open time by alphaxalone has been inferred by fluctuation analysis and marked prolongation of single channel open and burst durations by pregnanolone has been reported (Barker *et al.* 1987; Mistry & Cottrell, 1990). Since steroid effects on the GABA receptor were not blocked by the benzodiazepine receptor antagonist Ro 15-1788, their actions do not appear to be mediated through the benzodiazepine receptor (Cottrell *et al.* 1987). Pregnenolone sulphate antagonized GABA responses in a fashion similar to picrotoxin, suggesting at least an interaction through the picrotoxin binding site although multiple binding sites for pregnenolone sulphate have been proposed (Majewska *et al.* 1988).

The microscopic or single-channel kinetic mechanisms for the regulation of the

GABA receptor channel gating (opening and closing) by neurosteroids remain unclear. Even if neurosteroids and barbiturates prove to have separate binding sites, the basis for the observation that some steroids and barbiturates appear to act in a similar fashion is unknown. To further study neurosteroid mechanism of action of GABA receptors, single channel GABA receptor currents modulated by AND and PRE were recorded from excised outside-out patches obtained from mouse spinal cord neurones in culture and evaluated using single channel analysis techniques.

METHODS

Cell culture

To obtain spinal cord neurone cultures, timed pregnant mice were anaesthetized using CO₂ narcosis and then their necks were fractured. The 12- to 14-day-old fetuses were removed and decapitated. The spinal cords were dissected from the fetuses and were mechanically dissociated to yield a single cell suspension and grown in culture medium as described previously (Macdonald, Rogers & Twyman, 1989a). Cultures were maintained for 2–5 weeks prior to being used in these experiments.

Solutions

Thirty minutes prior to the first recording, the medium used to grow and maintain the cultures was exchanged for 1.5 ml of extracellular solution which consisted of the following (mM): 142 NaCl, 8.1 KCl, 1 CaCl₂, 6 MgCl₂, 10 glucose, 10 HEPES, (pH ~ 7.4). A high concentration of Mg²⁺ was used in the extracellular solution to stabilize excised patches. The solution used in the micropipettes contained (mM): 153 KCl, 1 MgCl₂, 10 HEPES, 5 EGTA, 1 NaOH, 2 KOH, (pH ~ 7.4). This combination of extracellular and micropipette solutions resulted in a chloride equilibrium potential (E_{Cl}) of about 0 mV and a potassium equilibrium potential (E_K) of about -75 mV. All recordings were performed at room temperature (21–23 °C).

GABA and steroid application

A 1 mM-GABA (Sigma) stock solution in distilled water was prepared prior to experiments and frozen in 1 ml aliquots. The stock solution of GABA was diluted with extracellular solution to a final concentration of 2 μ M on the day of each experiment. AND and PRE (Sigma) were dissolved in ethanol and serially diluted in external bathing solution on the day of experiments. Final concentrations of ethanol was not greater than 0.1% and these concentrations of ethanol did not affect the GABA currents. A mixture of GABA or GABA plus steroid was applied to the patch for at least 60 s via pressure ejection micropipettes that were kept out of the bath and moved to within 10 μ m of patches only during the time of each application. Three or four independent pressure ejection pipettes connected to separate and equal pressure lines contained GABA or GABA plus steroid. Pressure ejection pipettes were constructed to have nearly the same orifice diameter (15–20 μ m). GABA alone was applied first in at least two sequential applications to establish stable responses to GABA. Applications were separated by at least 1 min. If possible, all concentrations of GABA and a particular steroid were applied to each patch recording prior to patch disruption. The lowest concentration of steroid was applied first. Responses to AND or PRE were obtained from separate patches.

Current recording

Pressure ejection and recording micropipettes were constructed as previously described (Macdonald *et al.* 1989a). Recording micropipettes were coated with polystyrene Q-dope (GC Electronics, Rockford, IL, USA) to reduce capacitance. Recordings were obtained using a model L/M EPC-7 amplifier (List Medical Instruments, Darmstadt). Single-channel currents were low-pass filtered (3 db at 10 kHz, eight-pole Bessel filter) and simultaneously recorded on a video cassette recording (VCR) system (Sony SL-2700) via a digital audio processor (sony PCM-501ES, modified to 0 to 20 kHz, 14-bit 44 kHz sampling) and on a chart recorder (Gould Inc.).

Single channel current analysis

Data were accepted for analysis if only rare multiple openings (no evidence of more than three simultaneous openings) were detected during that application of GABA. Analysis was performed

using techniques previously described and are summarized below (Macdonald *et al.* 1989*a*; Twyman & Macdonald, 1991; Twyman, Green & Macdonald, 1992). Single-channel data were played back from the VCR system, digitized (20 kHz, 50 μ s/point, 14 bit, 40·96 points/pA) with a low-pass (3 dB at 2 kHz), eight-pole Bessel filter interposed, and analyzed by computer using locally written analysis programs. System dead time and rise time were 70 and 130 μ s, respectively. System dead time was determined by measuring the amplitude at the system output (after filtering but prior to digitization) of pulses of known amplitude inputted at the amplifier headstage. The duration of a pulse where the measured output peak amplitude was 50% of the input amplitude was the system dead time. System rise time was measured at the system output and was the time required for a square pulse to increase from 10 to 90% of its true amplitude. Amplitude distributions of channel openings were determined prior to temporal analysis of channel current. The amplitude of those channel openings longer than twice the system rise time could be reliably identified and only two primary current amplitudes were found in these data. Details of the method of channel detection for multiple conductance levels have been previously described (Twyman & Macdonald, 1991). To summarize briefly, channel openings to three possible amplitudes and their respective closings could be detected independently using the 50% threshold crossing method for the three independent current amplitudes. These independent current amplitudes corresponded to channel cord conductances of about 20, 28 and 42 pS. To be accepted as a valid opening, average current amplitude during an opening had to be within a specified window (approximately 1·5–2 standard deviations of the noise variance) around the channel current amplitude. Windows were non-overlapping.

Durations of detected closings and openings less than twice the system dead time were counted as unresolved open and closed times, respectively. Detected openings with a duration greater than twice the system dead time but less than two and a half times the system rise time (sampled points from 150 to 350 μ s) were compensated for amplitude distortion due to the limited system response (Twyman & Macdonald, 1991). Such compensation had little effect on open duration time constants and relative proportions of the exponential components since open duration histograms were fitted starting from bins that were greater than twice the system rise time. However, the number of openings detected at each amplitude level was affected by the compensation, and those openings with short time constants were primarily affected. For example, if all of the openings originated from an open state with a time constant of about 0·5 ms, the compensation could affect about 33% of the detected openings. In practice, the compensation usually affected about 5% of the briefest openings of the main-conductance level evoked by 2 μ M-GABA, but could affect a greater proportion of the openings to the sub-conductance level since those openings were generally shorter than those of the main-conductance level. In this study, potential openings with amplitudes smaller than the amplitude of sub-conductance level openings were counted as closures while multiple and ambiguous openings were rejected (deleted). After detection, the output contained condensed data consisting of a series of open and closed durations and their amplitudes. The data then could be analyzed selectively for main- or sub-conductance level openings or a combination of conductance levels.

Using locally written programs, open durations were placed into frequency histograms using linear and logarithmic binning, closed durations were placed into frequency histograms using logarithmic binning, and burst durations were placed into frequency histograms using linear binning. Linear frequency histograms were binned to minimize bin promotion errors according to methods previously described (McManus, Blatz & Magleby, 1987; Macdonald *et al.* 1989*a*). For linear histograms, open durations were binned into 0·1 ms bins with a range of 0·4 to 30 ms, and burst durations were binned into 0·5 ms bins with a range of 0·5 to 150 ms. Logarithmic binning used a logarithmic time axis and a square-root ordinate transformation (Sigworth & Sine, 1987). For logarithmic histograms, open durations were binned into 50 bins and 25 bins/decade resolution with a 400 μ s lower limit. Closed durations were binned using 50 bins and 10 bins/decade resolution with a 300 μ s lower limit. Exponential curve fitting to determine the maximum likelihood estimates of time constants and areas was performed using locally written programs described previously (Macdonald *et al.* 1989*a*; Twyman, Rogers & Macdonald, 1990; Twyman & Macdonald, 1991; Stat Library, IMSL, Inc., Houston, TX, USA). Error ranges for the estimates were calculated using maximum likelihood ranges ($m = 2$) which corresponded to about a 95% confidence interval. The number of significant exponential components was determined by fitting with increasing numbers of exponentials until (1) the χ^2 test of the estimated fit and the data was

within the 95% confidence interval for accepting the null hypothesis (no difference between the estimated fit and data) and/or (2) the maximum likelihood estimate was no longer improved greatly by the addition of additional exponential components (McManus & Magleby, 1989). Distributions of open, closed or burst durations were fitted over the same histogram ranges for GABA and each concentration of steroid.

The open and closed durations reported in this study were observed durations. Due to the presence of unresolved or missed events, the observed event durations can be greater than the true durations. Correction of average open and closed duration for missed events can be calculated from a detailed kinetic model (Blatz & Magleby, 1986). However, a simple correction of average open duration for missed short openings, for example, can be obtained by re-estimating the average open duration from the exponential function fits of the open duration distributions. Corrected average open duration was calculated by taking the sum of the relative area (a) of each exponential component in the open duration histogram multiplied by the time constant (τ) of the component (corrected average open duration = $a_1\tau_1 + a_2\tau_2 + a_3\tau_3$). Corrected average burst duration can be calculated similarly. Since burst durations were longer than open durations, the proportion of missed short bursts was less than that for short openings. Consequently, the correction for missed short bursts would have less effect. However, very long bursts could be rejected by the analysis methods. Since the number of actual channels per patch was unknown, longer bursts compared to shorter bursts have a greater likelihood of being interrupted by the opening of a second channel and therefore rejected by the analysis routine. Thus, correction of the average burst duration using the time constants derived from frequency distributions can result in an average burst duration longer than the measured average burst duration.

Since the data contained a small number of multiple simultaneous main-conductance level openings and openings to multiple conductance levels and since the different conductance levels may have represented openings of different channels, it was difficult to determine unambiguously which closed durations represented the 'main-conductance level closed durations' or 'sub-conductance level closed durations'. Most of the analysis of this study was concentrated on closed durations between main-conductance level openings. With this analysis, it assumed that closed durations between main-conductance level openings were gated independently of other conductance levels, and thus, non-main-conductance level openings were counted as closures while periods containing multiple openings were rejected (deleted). In addition, closed durations between all openings (multiple, main- and sub-conductance levels) and closed durations between sub-conductance level openings (which assumed that sub-conductance level openings were independent of main-conductance openings) were examined.

Total average current was defined as the average current evoked over time per agonist application and included contributions from multiple simultaneous openings and openings to all conductance levels. Steroid enhancement of total average current was calculated from all patches containing paired control and drug responses. Although the absolute numbers of channels in the patches were unknown, the increases in total average current were calculated as percentage changes from paired controls. Due to desensitization, these changes certainly did not represent changes in peak current. Measurements of peak current will have to await more rapid application techniques.

Kinetic properties were analyzed using pooled data from multiple patches. Pooled data permitted more stable curve fitting of open-, closed- and burst-duration frequency histograms. Data from separate patches with large numbers of openings showed similar results compared to the pooled data.

Average data are presented as the mean \pm s.d. unless otherwise indicated.

Treatment of patches with multiple active channels

The presence of multiple active channels in the patch may have affected measured open and burst durations. The effects would be more significant in patches with greater opening frequency and percentage time open. These problems were addressed by applying a method for estimating stochastic properties of channel openings in patches containing two channels (Colquhoun & Hawkes, 1990). The approximation method was used in estimating the average duration of runs of single (serial non-multiple) openings in a patch containing two active channels. Using the measured average open duration and percentage time open, data sets with runs that were three times the estimated average length of runs of single openings between double openings were identified. The runs from these data sets were likely ($P = 0.05$) to be representative of the activity of a single channel. Kinetic properties obtained from these runs were compared to those from the pooled data.

For 2 μM -GABA applications, three times the estimated average run length of single openings was often greater than the 60 s GABA application. Thus, for some 2 μM -GABA applications, runs were accepted if no simultaneous multiple openings were observed during the application and the application duration was greater than twice the expected run length of single openings.

Definition of bursts

Bursts may be defined as openings or groups of openings separated by relatively long closed periods. For the purpose of quantitative analysis, a critical closed time, t_c , was chosen such that all opening separated by closure less than t_c belonged within a burst, and bursts were separated by closures greater than t_c . A modified form of the equal proportion of misclassifications method (Macdonald *et al.* 1989a) was used to determine the t_c for the data in this study.

RESULTS

Conductance properties

Following hyperpolarization of outside-out patches to -75 mV, rare spontaneous channel openings were observed (not illustrated). Application of GABA (2 μM) evoked bursting currents in 46/56 patches (Fig. 1A), and the single-channel activity was steroid concentration dependent (Fig. 1B and C). Only patches with reproducible responses to the initial applications of GABA (also see stationarity tests below) and rare multiple simultaneous open channel currents (three or less simultaneous main-conductance level openings) were used for kinetic analysis.

Similar to that previously reported (Macdonald *et al.* 1989a), channel openings evoked by GABA occurred with primarily two current amplitudes which corresponded to at least two cord conductance levels (Fig. 1A). The larger conductance level (Fig. 1A, **) was recorded more frequently than the smaller conductance level (Fig. 1A, *). Current amplitudes evoked by GABA alone (2 μM) averaged 2.05 ± 0.23 pA and 1.47 ± 0.12 pA at -75 mV ($n = 46$ patches). The single channel currents reversed at about 0 mV. For control recordings exposed to GABA alone, channel cord conductances from all patches were 27.3 ± 3.1 pS and 19.6 ± 1.6 pS for the large and small conductance levels, respectively. GABA receptor channel current amplitudes were unaltered by the steroids (Fig. 1B and C) and averaged 2.09 ± 0.15 pA and 1.48 ± 0.10 pA and corresponded to cord conductances of 27.9 ± 2.0 pS and 19.6 ± 1.3 pS.

The larger or main-conductance level comprised 88.1% of detected openings when GABA was applied alone. Since it was larger than the sub-conductance level, the main-conductance level was responsible for 96.4% of the total average current evoked. As a group, the proportions varied little in the presence of the steroids with the larger or main-conductance level comprising 87.7% of detected openings and 96.6% of the total average current evoked. When currents in the presence of AND or PRE were analyzed separately, there were no differences in the percentages of main-conductance level openings when compared to each other or to GABA alone.

Transitions between main- and sub-conductance levels

Direct transitions between main-conductance level and sub-conductance level openings (without an observed closing) and vice versa were occasionally observed (not illustrated). Openings to the sub-conductance level were infrequent and were generally interposed between main-conductance level openings, but occasional prolonged series of sub-conductance level openings were observed (not illustrated).

For GABA alone, the percentage of transitions of the main-conductance level to the sub-conductance level relative to all detected openings and vice versa were 0.17 and 0.19%, respectively (Table 1). Similar findings of approximately equal percentages of transitions were found for currents enhanced by AND and PRE.

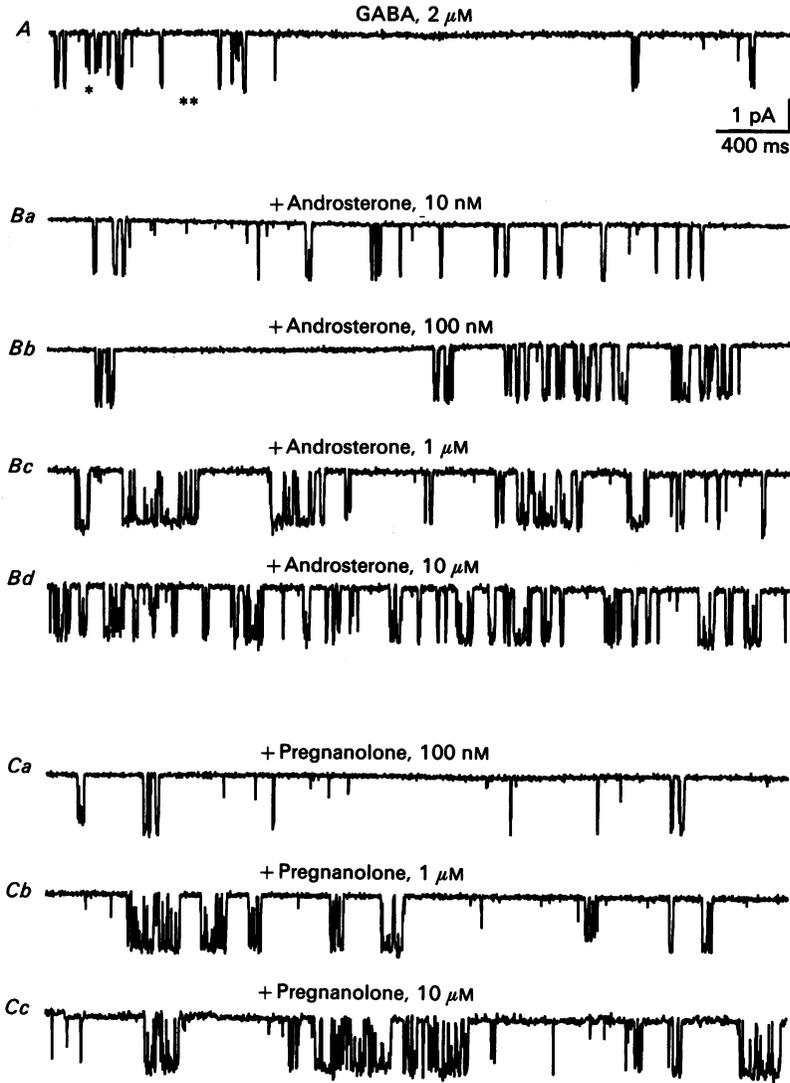


Fig. 1. *A*, GABA receptor single-channel currents were altered in the presence of the neurosteroids androsterone (*B*) and pregnanolone (*C*). GABA ($2\ \mu\text{M}$) evoked single inward (down-going) bursting currents with at least two amplitudes when outside-out patches were voltage clamped at $-75\ \text{mV}$. Openings to the larger $2.05\ \text{pA}$ ($28\ \text{pS}$) level (**) occurred more frequently than to the smaller $1.47\ \text{pA}$ ($20\ \text{pS}$) level (*). Tracings in *A* and *B* were obtained from the same patch. Tracings in *C* were obtained from a different patch. Tracings are shown at low time resolution, and the amplitude of brief openings was attenuated due to the graphics plotting routine which plotted the average of 8192 consecutive 64 sample point segments. Time and current calibrations apply throughout.

Compared to GABA, the percentages of transitions were not greatly different for GABA receptor currents in the presence of AND or PRE and ranged from 0.08 to 0.21% (Table 1).

Stationarity of the single channel properties

At low time resolution, the steroids produced visually apparent changes in the single-channel kinetic properties including total current evoked, percentage time

TABLE 1. Steroid regulation of GABA receptor channel properties. Observed transitions between conductance levels

	28 to 20 pS (%)	20 to 28 pS (%)
GABA, 2 μ M	0.17	0.19
+ AND, 10 nM	0.08	0.08
+ AND, 100 nM	0.17	0.16
+ AND, 1 μ M	0.10	0.08
+ AND, 10 μ M	0.14	0.18
+ PRE, 100 nM	0.14	0.17
+ PRE, 1 μ M	0.13	0.10
+ PRE, 10 μ M	0.21	0.14

Percentages of transition between main-conductance (28 pS) and sub-conductance (20 pS) levels for GABA alone and in the presence of androsterone (AND) or pregnanolone (PRE) were equal for transitions in both directions. Percentages are given for the total number of openings detected.

open and channel-opening frequency (Figs 1, 2Aa and 2Bb). At increased time resolution, samples of channel activity revealed an apparent prolongation of average channel open and burst durations in the presence of 1 μ M-AND compared to GABA alone (Fig. 2).

To examine the time-dependent properties of patches exposed to a constant concentration of agonist, the data at each concentration were divided into six consecutive 10 s epochs and analyzed from 1 s after the beginning of GABA application (Fig. 3). Average current evoked, percentage time open, opening frequency and average open duration for each application were calculated. The total average current evoked and the average percentage time open in the main-conductance level decreased with time in the presence of GABA. With the addition of AND or PRE (not illustrated), the total average current evoked and the average percentage time open in the main-conductance level also decreased with time, but remained increased compared to GABA alone. Opening frequency also decreased with time from the beginning of GABA application. Channel opening frequencies declined with time to a plateau, but also remained increased in the presence of the steroids compared to GABA. The decline in opening frequencies was observed during a steady application of agonist at the patch. During a 60 s application of GABA, opening frequency decreased about 8.9% every 10 s. The decreases in opening frequency over time were less apparent in the presence of AND and were AND concentration dependent. Opening frequency decreased about 8.6, 1.0 and 1.8% every 10 s for AND at concentrations of 100 nM, 1 μ M and 10 μ M, respectively.

In contrast, average open durations remained relatively stable during the agonist application and demonstrated equilibrium stationarity of open kinetic state(s) of the

GABA receptor main-conductance level. In the presence of the steroids, the average channel open durations were increased in a steroid concentration-dependent manner. Thus, in the presence or absence of steroid, the open state properties were unchanged with time, and the initial decreases in average current and percentage time open

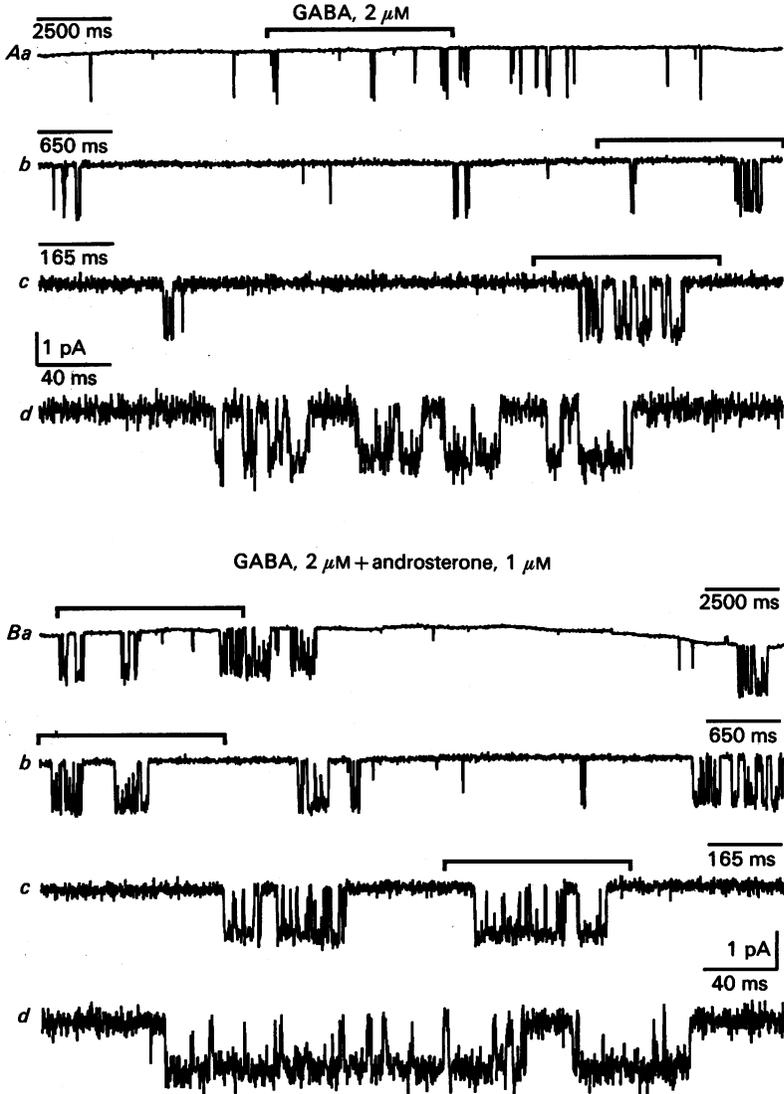


Fig. 2. GABA-evoked (*A*) and androsterone-enhanced (*B*) GABA receptor single-channel currents are shown at increased time resolution. Tracings represent samples of channel activity selected to demonstrate overall behaviour of the evoked responses. Data are from the same patch. Portions of channel activity under the bracketed lines are shown at increased time resolution in the tracing directly below. Current calibration applies throughout.

following exposure to GABA were due primarily to decreased channel opening frequency. This process may have been due to desensitization. It is noted that the desensitization process(es) was not studied and fast agonist application techniques were not used to evaluate rapid desensitization.

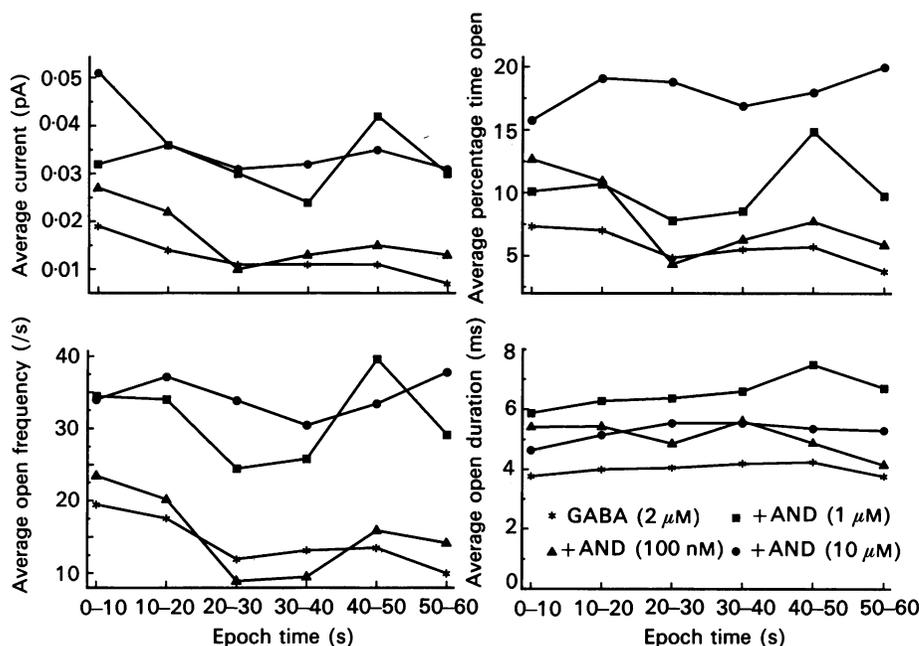


Fig. 3. Some properties of the GABA receptor main-conductance level were time dependent. In the presence of 0–10 μM-androsterone (AND), GABA receptor main-conductance level average current, opening frequency and percentage time open declined with time, but average open duration was stationary. Data were analysed in six consecutive 10 s epoches from 1 s after the beginning of agonist application. GABA (2 μM) average current, opening frequency, percentage time open and open duration were increased in the presence of androsterone (AND, 0.1–10 μM).

Average open durations of openings to the sub-conductance level also remained relatively stable during agonist application (not illustrated). However, the low numbers of openings to the sub-conductance level per 10 s epoch resulted in a large degree of variability of opening frequency and percentage time open, and therefore, conclusions about the stationarity of opening frequency or percentage time open of the sub-conductance level could not be reached.

Steroid enhancement of GABA receptor currents

AND and PRE increased GABA receptor current in a concentration-dependent fashion (Fig. 1). In the first 60 s of application, GABA (2 μM) evoked an average total current of 103 fA per application (n = 141). In those patches where paired control and steroid responses were obtained, AND increased total average current in the first 60 s of application by 25% at a concentration of 10 nM (n = 6) and by 318% at 10 μM (n = 9), while PRE increased GABA receptor current by 55% at 100 nM (n = 9) and

by 268% at 10 μM ($n = 6$). The slopes of log-log plots of the increases in GABA receptor total average current at equilibrium *versus* steroid concentration were 0.56 for AND over the range of 10 nM to 10 μM and 0.74 for PRE over the range of 100 nM to 10 μM (not illustrated).

These increases in currents were similar to the increases in the percentage time open of the main-conductance level found for pooled data which included data from non-paired applications (Table 2A). For all of the applications of GABA alone in all of the patches, the percentage time open in the main-conductance level was 3.0% ($n = 141$). For the pooled applications, the percentage time open was increased in a concentration-dependent fashion by the steroids. GABA receptor currents were increased by AND from 31% at 10 nM to 375% at 10 μM . PRE increased the percentage time open of the GABA receptor channel from 82% at 100 nM to 345% at 10 μM .

Steroid-dependent kinetic properties of the main- and sub-conductance levels

Kinetic properties of openings to the main-conductance level of GABA receptor current were steroid concentration dependent. Although during the first 60 s of GABA application the observed opening frequency decreased with time, the frequencies of main-conductance level opening from pooled agonist applications were increased by AND and PRE compared to GABA alone. GABA evoked an average of 8.7 openings/s for the main-conductance level. In the presence of AND, opening frequency increased from 22% at 10 nM to 251% at 10 μM . Similar concentration-dependent increases in opening frequency of the main-conductance level were found for PRE. For the main-conductance level, GABA (2 μM) evoked channel openings with an average duration of 3.92 ms (2.77 ms when corrected for undetected openings). Average channel open duration was increased by the steroids in a concentration-dependent manner. AND increased the average GABA receptor channel duration from 4.47 ms (3.10 ms, corrected) at 10 nM to 6.87 ms (6.24 ms, corrected) at 1 μM , but decreased it to 4.92 ms (3.99 ms, corrected) at 10 μM . Similarly for PRE, the average GABA receptor channel open durations were increased at the lower concentrations (100 nM and 1 μM) and also decreased at the highest concentration (10 μM , Table 2A). Since the main-conductance level accounted for about 96% of the total receptor current, the enhancement of GABA receptor current by AND and PRE was due to an increase in average open duration and opening frequency of openings to the main-conductance level.

Similarly, kinetic properties of openings to the sub-conductance level were steroid concentration dependent (Table 2B). For GABA alone, the percentage of time open in the sub-conductance level was 0.32%. The percentage times open in the sub-conductance level were also increased by the steroids but only to a moderate degree compared to the main-conductance level. The percentage time open was increased by only 70–80% at the highest steroid concentrations. For sub-conductance level opening that could be reliably detected, GABA evoked an average of 1.7 openings/s. In the presence of AND, opening frequencies of the sub-conductance level were increased 57% at 10 nM and 94% at 10 μM . Similar moderate increases in opening frequencies of the sub-conductance level were found for PRE. For the sub-conductance level, GABA (2 μM) evoked channel openings with an observed average duration of 1.94 ms (1.00 ms, corrected). AND increased the average channel open

TABLE 2. Steroid regulation of GABA receptor open and closed properties

	Percentage time open ($\Delta\%$)	Opening frequency ($\Delta\%$)	Average open duration (ms)	Corrected average open duration (ms)	Average closed duration (ms)	Number of openings	Number of patches
A. Main-conductance level (28 pS)							
GABA, 2 μ M	—	—	3.92	2.77	115	92030	46
+AND, 10 nM	31	22	4.47	3.10	94	12342	6
+AND, 100 nM	103	36	4.96	4.88	85	12224	5
+AND, 1 μ M	198	74	6.87	6.24	66	39647	15
+AND, 10 μ M	375	251	4.92	3.99	33	59224	9
+PRE, 100 nM	82	41	5.07	3.89	82	13157	9
+PRE, 1 μ M	152	72	5.59	4.13	67	25964	9
+PRE, 10 μ M	345	211	5.33	3.74	37	10769	6
B. Sub-conductance level (20 pS)							
GABA, 2 μ M	—	—	1.94	1.00	582	13826	
+AND, 10 nM	42	57	2.04	1.24	377	1547	
+AND, 100 nM	76	69	2.20	1.40	350	2373	
+AND, 1 μ M	75	43	2.39	1.58	414	44657	
+AND, 10 μ M	70	94	2.08	1.19	305	7120	
+PRE, 100 nM	2	4	2.11	1.44	569	2155	
+PRE, 1 μ M	70	67	2.30	1.33	354	4716	
+PRE, 10 μ M	80	34	2.66	2.00	442	2516	

Androsterone (AND) or pregnanolone (PRE) open and closed properties of the GABA receptor channel were concentration dependent. Properties were derived from detected openings and closings (see Methods). Properties represent pooled results from multiple patches. Number of openings indicates total number of pooled openings.

duration from 2.04 ms (1.24 ms, corrected) at 10 nM to 2.39 ms (1.58 ms, corrected) at 1 μ M but decreased it to 2.08 ms (1.19 ms, corrected) at 10 μ M. Similarly, PRE increased average durations of the GABA receptor channel sub-conductance level. Thus, average open durations of the sub-conductance level were also increased in the

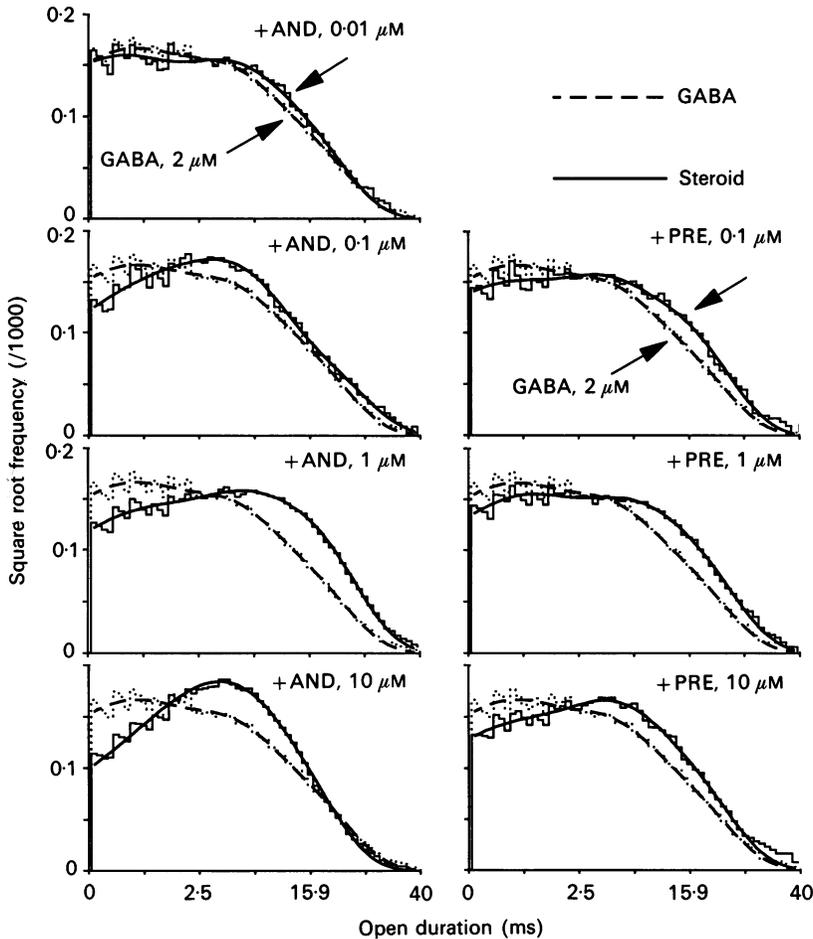


Fig. 4. Logarithmic binned open-duration frequency distribution histograms were neurosteroid dependent. Logarithmic histogram distributions were plotted with square root transformed ordinate values (see Methods). Histograms were fitted best with sums of three exponential functions and curves were drawn according to the fits (see text). The open-duration distribution for GABA is shown in each panel for each concentration of androsterone (AND) and pregnanolone (PRE). Note the decreased area of shorter open durations in the presence of the steroids compared to GABA alone.

presence of the steroids, but since the numbers of sub-conductance openings were low and their average open durations were shorter than the main-conductance level, the contribution of the sub-conductance level to the total steroid enhanced current was small.

Frequency distributions of open durations

To determine the basis for the increases in average open durations by the steroids, open durations were collated into frequency histograms. For openings to the main-conductance level, open durations were shifted to longer durations in the presence of

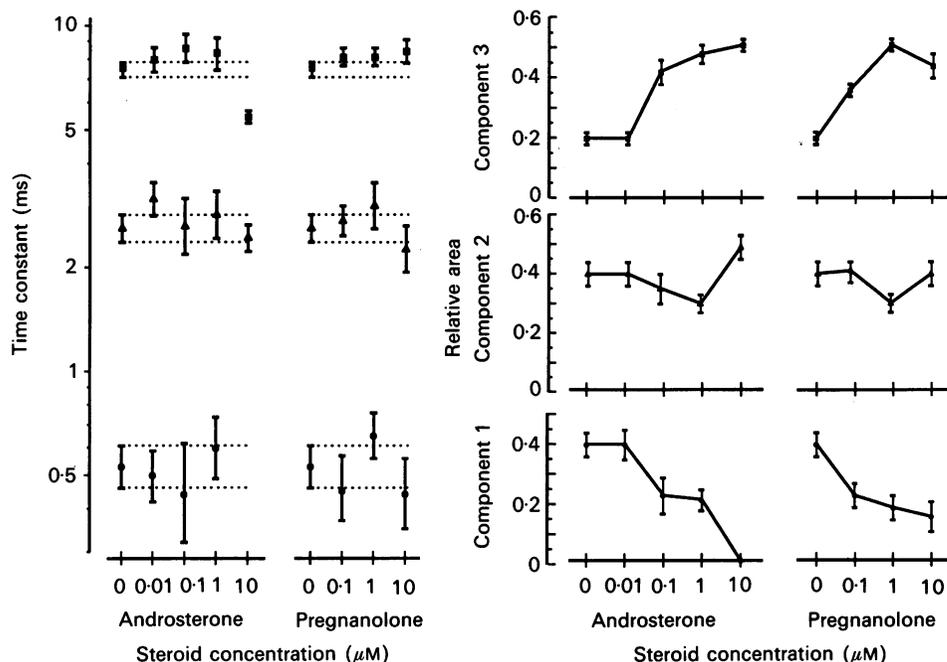


Fig. 5. Time constants and relative areas of open frequency distributions for the GABA receptor main-conductance level were neurosteroid concentration dependent. Components 1 to 3 correspond to function with the shortest to the longest time constants, respectively. Error bars represent likelihood intervals ($m = 2$) or approximately 95% confidence intervals. The dashed lines across the time constant graph represent the upper and lower limits of the time constant estimates for GABA.

steroids (Fig. 4). Open-duration frequency histograms for GABA ($2 \mu\text{M}$) were fitted best with a sum of three exponential functions. The exponential components were designed 1 to 3 for the shortest to the longest time constants, respectively (Fig. 5). Results from the logarithmic binned fits of open durations in the steroid-enhanced data sets were not greatly different from the results found in the linear-binned fits. The time constants from linear- or logarithmic-binned histogram fits were 0.53, 2.6 and 7.6 ms for components 1, 2 and 3, respectively, and were similar to those reported previously (Macdonald *et al.* 1989a; Twyman *et al.* 1990). Slight differences in the time constants may be accounted for by improvements in recording resolution. At all of the concentrations of the steroids except $10 \mu\text{M}$ -AND, the open-duration frequency histograms of main-conductance level openings were fitted best with sums of three exponential functions (Fig. 5). The GABA receptor main-conductance level open

time constants were not altered significantly by the steroids, but the steroids increased the relative proportion of the longer time constants. As AND and PRE concentrations were increased, there were further increases in the proportions of the longest open time constant. Thus, the steroids increased the average open duration

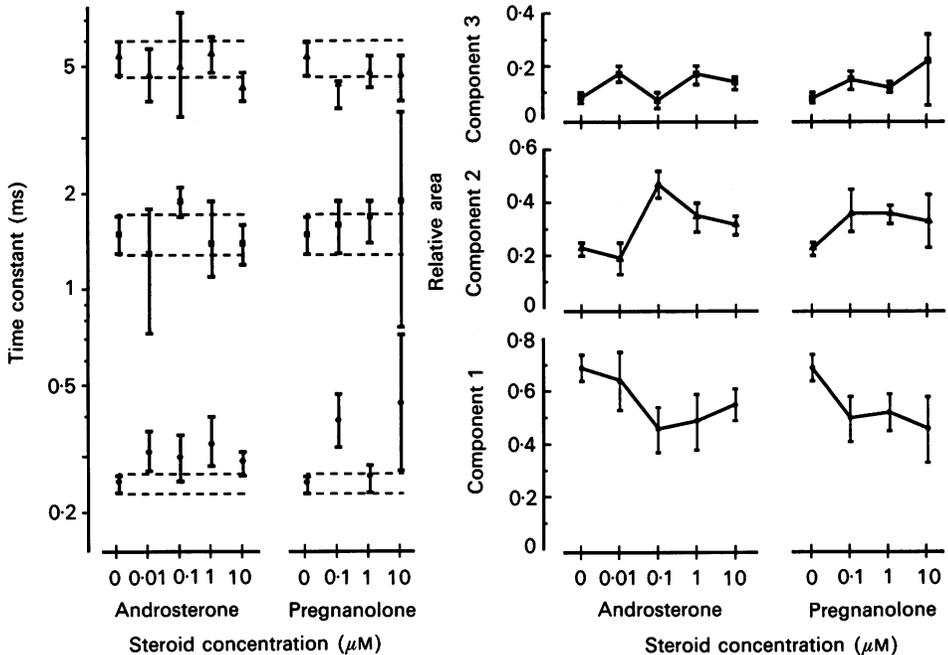


Fig. 6. Time constants and relative areas of open frequency distributions for the GABA receptor sub-conductance level were neurosteroid concentration dependent. Components 1 to 3 correspond to functions with the shortest to the longest time constants, respectively. Error bars represent likelihood intervals ($m = 2$) or approximately 95% confidence intervals. The dashed lines across the time constant graph represent the upper and lower limits of the time constant estimates for GABA.

of the main-conductance level of the GABA receptor by shifting the proportion of openings from short open dwell times to the longer open dwell times. However, at the highest concentration of AND ($10 \mu\text{M}$), a sum of only two exponentials was required for an optimum fit. Both time constants were longer than the shortest time constants in the other data sets. Both time constants appeared to correspond to components 2 and 3 but were slightly shorter than those found for GABA and for the other AND concentrations.

For openings to the GABA receptor sub-conductance level, the open-duration frequency histograms were shifted slightly to longer durations in the presence of the steroids, although the shifts in the normalized open-duration frequency histograms were not as readily apparent as for the main-conductance level (not illustrated). The open-duration frequency histograms were fitted best with a sum of three exponential functions (Fig. 6). The time constant for GABA were 0.25, 1.5 and 5.4 ms for components 1, 2 and 3, respectively. Like the main-conductance level, three

exponential component were resolved, but these time constants were shorter than those found for the main-conductance level. The GABA receptor sub-conductance level open time constants were more difficult to estimate than those for the main-conductance level and were not altered significantly by the steroids. Similar to the main-conductance level, both steroids tended to increase the proportion of the longer time constants. However, estimates of the relative areas were difficult, and the maximum likelihood estimates of some of the ranges overlapped those of GABA. Although more weakly supported due to the difficulties in component estimation, the basis for the increased GABA receptor channel average open durations of the sub-conductance level by the steroids was similar to the main-conductance level. Unlike the main-conductance level, however, the open time constants for the sub-conductance level were unaltered by 10 μM -AND.

Frequency distributions of closed durations

Closed durations were evaluated using three different assumptions (see Methods). For each closed-duration analysis method, the closed durations were widely distributed (microseconds to seconds). In each, closed durations shorter than 200 μs could not be accurately classified as closures to baseline or to a sub-conductance level. For GABA (2 μM), the average closed duration between main-conductance level openings was 115 ms (Table 2A). The average closed duration between all detected openings to the main- or sub-conductance levels was 95 ms. The average closed duration between sub-conductance level openings was 582 ms (Table 2B). In the presence of the steroids, the average closed durations between all detected openings to the main- and sub-conductance levels, between main-conductance levels and between sub-conductance levels were decreased compared to GABA alone and these averages were steroid concentration dependent.

Since the total number of openings was dominated by main-conductance openings, the distributions of closed durations between main-conductance level openings were not greatly different compared to closed duration between all conductance level openings at each steroid concentration. The frequency distributions for closed durations between main-conductance level openings revealed a relative increase in the areas of exponential functions with shorter time constants (Fig. 7). The time constants for components 1 to 3 in the main-conductance level closed-duration distributions at all concentrations of steroid were similar to those found for GABA (Fig. 8). For GABA, the time constants for components 1 and 2 were 0.22 and 1.42 ms, respectively. These two brief time constants were not greatly different from those reported previously for low concentrations of GABA (Macdonald *et al.* 1989a). In the presence of the steroids, the average of the time constants for components 1 and 2 were 0.24 ± 0.03 and 1.45 ± 0.14 ms, respectively. The component 3 time constant for GABA was 7.5 ms and the average time constant in the presence of the steroids was 7.5 ± 2.0 ms. The time constants found for component 4 were somewhat variable and occasionally were found to be slightly shorter for the steroids than for GABA. The significance of the longer time constants was unclear since the contributions due to multiple channels or desensitization were unknown. Also, the relative contribution to the area of the longest time constant of the closed distributions was small and ranged from 0.001 to 4% for component 6. The relative

contribution of each of the closed-duration time constants to the total area of the closed-duration distribution was increased for the shorter time constants (Fig. 7). The proportion of the closed-distribution areas due to components 1, 2 and 3 were increased from 68 to $74 \pm 5\%$ in the presence of steroids. These results indicated that

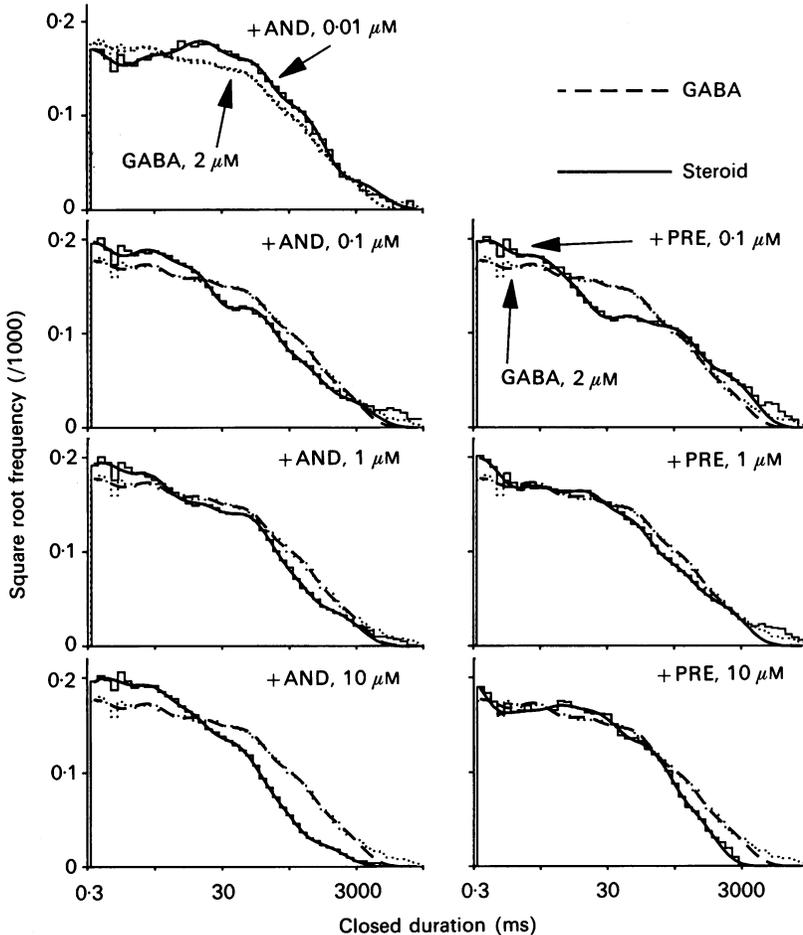


Fig. 7. Logarithmic binned frequency distribution histograms of the GABA receptor channel-closed durations between main-conductance openings were neurosteroid dependent (see Methods). Histograms were fitted best with sums of five to six exponential functions and curves were drawn according to the fits (see text). Closed-duration distribution for GABA are shown in each panel for each concentration of androsterone (AND) and pregnanolone (PRE).

the leftward shift in the area of the closed-duration distributions in the presence of the steroids was due to an increase in the relative number of closings with shorter time constants and not due to a change in the short closed time constants. This also indicated that the increase in opening frequencies observed for the steroids was in part due to an increase in the number of short closings.

The distributions of closed durations between sub-conductance level openings showed more long-duration closures compared to those found for closures between all openings and those between main-conductance level openings (not illustrated). Exponential components were difficult to estimate due to the lower numbers of

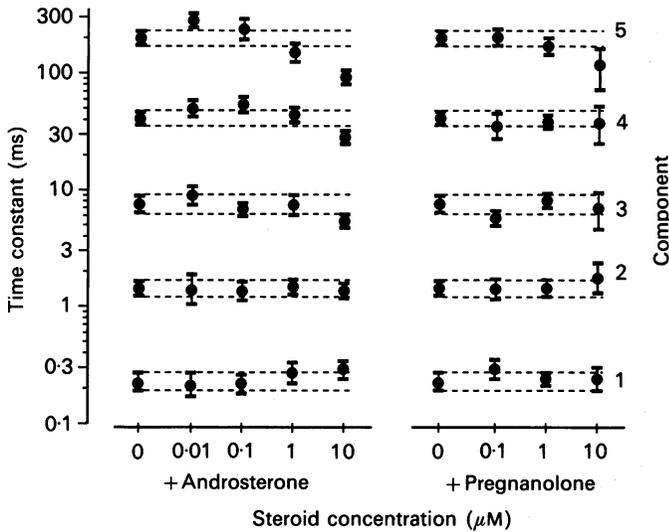


Fig. 8. Time constants of GABA receptor main-conductance level closed-duration frequency distributions were neurosteroid concentration dependent. Components 1 to 5 correspond to functions with the shortest to the longest time constants, respectively. Error bars for the time constants represent likelihood intervals ($m = 2$). The dashed lines across the time constant graph represent the upper and lower limits of the time constant estimates for GABA.

openings to the sub-conductance level. The time constants tended to be long since closed durations between sub-conductance level openings were generally longer because these openings tended to occur in isolation or were frequently interposed between main-conductance level openings. The significance of these long time constants was unclear.

Burst properties

A critical closed time (t_c) of 5 ms was previously described for the determination of the end of a burst evoked by low concentrations of GABA (0.5–5 μM) (Macdonald *et al.* 1989a). For these data, a t_c was chosen between closed components 2 and 3 such that bursts would contain intraburst closures of components 1 and 2. A similar value for t_c was found for GABA (2 μM) in these data. Since short closed durations were not altered greatly in the presence of the steroids, a t_c of about 4–5 ms was also found for the steroids.

GABA receptor channel burst properties were steroid concentration dependent (Table 3). GABA (2 μM) evoked bursts with average duration of 9.7 ms (8.2 ms when

corrected for undetected and rejected bursts). Average durations of GABA receptor channel bursts in the presence of the steroids were increased compared to GABA alone. Average burst durations increased with increased AND and PRE concentration but were decreased at the highest concentration (10 μM). The average number of openings detected within a burst evoked by GABA (2 μM) averaged 2.14

TABLE 3. Steroid regulation of GABA receptor burst properties.
Main-conductance level (28 pS)

	Average burst duration (ms)	Corrected average burst duration (ms)	Average openings per burst	Average intraburst closed duration (ms)	Number of bursts
GABA, 2 μM	9.7	8.2	2.14	1.2	43023
+ AND, 10 nM	8.8	9.4	1.76	1.2	7021
+ AND, 100 nM	13.1	13.7	2.32	1.3	5280
+ AND, 1 μM	18.1	19.2	2.40	1.2	16535
+ AND, 10 μM	14.4	14.2	2.55	1.2	23253
+ PRE, 100 nM	13.3	13.8	2.31	1.2	5688
+ PRE, 1 μM	13.5	13.8	2.18	1.1	11917
+ PRE, 10 μM	12.7	12.7	2.14	1.1	5032

Androsterone (AND) or pregnanolone (PRE) burst properties of the GABA receptor channel were concentration dependent. Burst properties were derived from detected bursts and openings and closings within bursts. Bursts were separated by closures greater than 5 ms. Properties represent pooled results from multiple patches. Percentage time open within a burst was calculated by taking the percentage of the total open duration in a burst divided by the total duration in a burst (total open duration plus total intraburst closed duration). Number of bursts indicate total number of pooled bursts.

and were increased slightly in the presence of the steroids. The average duration of closed periods within a burst evoked by GABA was 1.2 ms and varied little in those bursts evoked in the presence of the steroids. The invariance of intraburst closures suggested that the mechanism producing brief closures of the channel was unaltered by the steroids or that once the GABA receptor channel was activated, the kinetic process of fast re-opening of the channel following interruption by a brief closure was unchanged by the steroids. Thus, the primary effect on burst properties by the steroids was a concentration-dependent increase in average burst duration which was due primarily to the incorporation of longer duration openings into bursts.

Most sub-conductance level openings tended to occur in isolation as evidenced by the paucity of brief closures found between sub-conductance level openings. Thus, bursts of several sub-conductance level openings tended to be rare. Due to the low number of bursts of sub-conductance level openings, burst properties of openings to the sub-conductance level were not analysed.

Frequency distributions of burst durations

To determine the basis for the steroid-depending increases in average burst durations of the main-conductance level, burst durations were collated into linear-binned frequency histograms. Burst durations were shifted to longer durations in the presence of steroids (Fig. 9). Burst-duration frequency histograms were fitted best

with a sum of three exponential functions, designated 1 to 3 for the shortest to the longest time constants, respectively (Fig. 10). For GABA ($2 \mu\text{M}$), the time constants were 0.61, 4.1 and 25.0 ms for components 1, 2 and 3, respectively. The GABA receptor main-conductance level burst-duration time constants in the presence of the

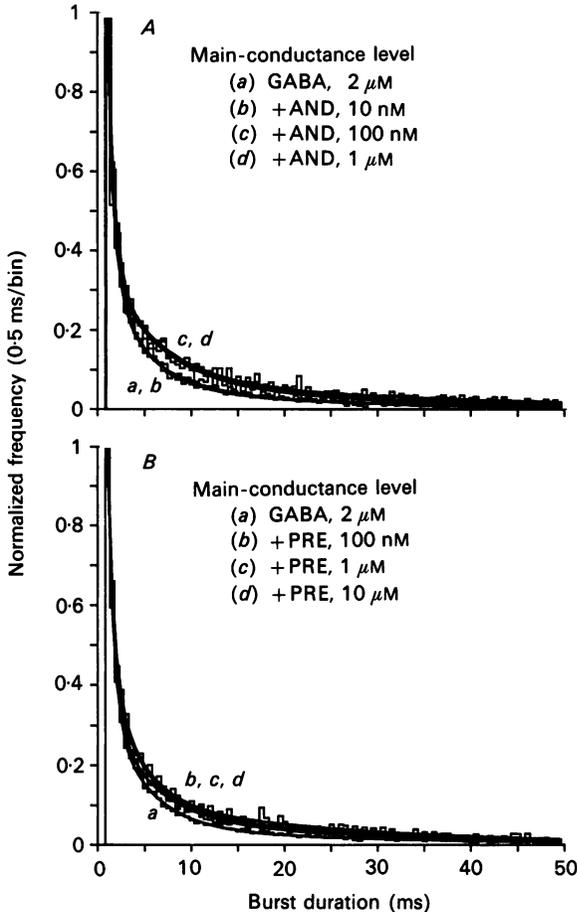


Fig. 9. Linear binned burst-duration frequency distribution histograms were neurosteroid concentration dependent. Burst durations were placed into 0.5 ms bins and displayed for clarity over a range of 1–50 ms. Distributions were normalized and overlaid to display relative frequency distributions. Histograms were fitted best with the sum of three exponential functions and curves were drawn according to the fits (see text). The distribution farthest to the left in each panel corresponds to GABA and androsterone (panel A) and pregnanolone (panel B) progressively shifted burst durations to longer durations to the right.

steroids generally were about the same as those found for GABA alone. The time constants ranged from 0.54–0.73, 3.3–5.7 and 20.6–27.2 ms for components 1, 2 and 3 respectively. However, component 3 of 10 μM-AND was lower (17 ms) and the estimate of its ranges did not overlap the 95% confidence range for burst duration component 3 of GABA alone. Estimates of the relative proportions of the components

showed that the steroids generally increased the relative proportion of the component with the longest time constant and decreased the relative proportion of the components with the two shortest time constants.

Effects of multiple channels on kinetic properties

To evaluate the effects of multiple active channels on the kinetic properties, patches with runs of single (non-multiple) openings were identified according to

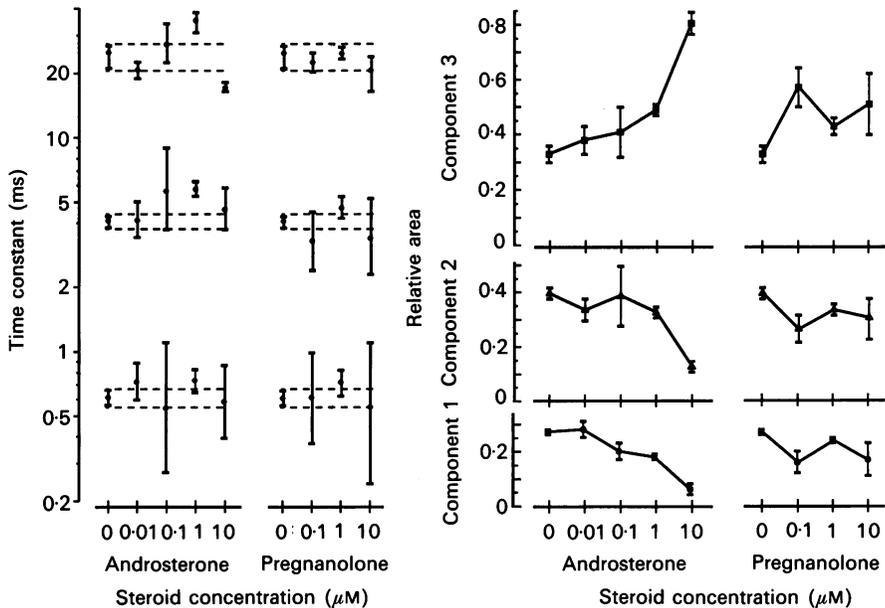


Fig. 10. Time constants and relative areas of burst-duration frequency distributions were neurosteroid concentration dependent. Components 1 to 3 correspond to functions with the shortest to the longest time constants, respectively. Error bars for the time constants represent likelihood intervals ($m = 2$). The dashed lines across the time constant graph represent the upper and lower limits of the time constant estimates for GABA.

criteria described in the Methods. The number of runs satisfying these criteria were few, and the numbers of openings available for analysis (1363–7873) were considerably fewer than that for the pooled data.

The open and burst properties of runs of single openings for AND (Table 4) were similar to those found for the pooled data (Tables 2A and 3). Measured opening frequency in the data sets containing runs of single openings increased with AND concentration from 1.9 to 7.1/s. With the exception of 10 μM-AND, average open and burst durations were increased in the presence of AND and were AND concentration dependent compared to GABA alone (Table 4). Average open and burst durations were longer in the data sets containing runs of single openings compared to the pooled data (Tables 2A and 3). In the pooled data, overlapping multiple openings and bursts containing multiple openings were rejected. Thus, the measured average open and burst durations in the pooled data would be expected to be shorter than those found for the runs of single openings. The observed differences were consistent

TABLE 4. Open and burst properties of runs of single openings. Main conductance level (28 pS)

	Average opening frequency (s^{-1})	Average open duration (ms)	Number of openings	Average burst duration (ms)	Average openings per burst	Average intraburst closed duration (ms)	Number of bursts
GABA, 2 μM	2.3	3.93	5263	9.8	2.1	1.1	2023
+ AND, 10 nM	1.9	4.72	7873	9.1	1.8	1.2	4534
+ AND, 100 nM	1.8	5.79	7402	16.0	2.5	1.2	2995
+ AND, 1 μM	4.0	7.71	5791	26.0	3.1	1.1	1883
+ AND, 10 μM	7.1	4.43	1363	14.2	2.7	1.3	502

Kinetic properties of runs of single openings were similar to the kinetic properties of channels from pooled data (see text).

with this expectation. In addition, data sets with longer average open and burst durations and greater opening frequencies and percentages time open were affected to a greater extent by the presence of multiple active channels. Similar results for kinetic properties in the presence of PRE were found.

DISCUSSION

Conductance

For patches held at -75 mV in symmetrical chloride solutions, the dominant or main-conductance level evoked by GABA was the 28 pS level and was similar to that previously reported for the GABA receptor channel (Bormann, Hamill & Sakmann, 1987; Macdonald *et al.* 1989*a*; Weiss & Magleby, 1989). Since the chord conductances of the main- and sub-conductance levels were unchanged in the presence of the steroids and since there were not significant shifts in the relative proportions of the main- and sub-conductance levels, the steroids did not appear to alter the conductance of the channel gated by GABA. Regulation opening frequency and duration of openings to the main-conductance level appeared to be the major mechanism for enhancement of GABA receptor current by AND and PRE.

Transitions between main- and sub-conductance levels

Observed direct transitions between the main- and sub-conductance levels may have resulted from actual direct conductance level transitions of the same receptor channel, coincident unresolved closure and re-opening of separate conductance level channels, or the apparent change in channel conductance due to rapid blocking and unblocking of a single channel. Due to limited system resolution, apparent transitions between conductance levels may have been due to the close approximation of closing of one channel with the opening of another channel with a different conductance. The calculation of the exact probability of such an occurrence would be difficult. However, a crude approximate likelihood of such occurrences can be calculated by assuming that all openings to the 20 pS level were independent of openings to the 28 pS level, and by assuming that closely approximated openings to independent 28 and 20 pS levels could occur with unobserved closures between them. Unobserved closures could occur when sequential closing and opening occurred within twice the system dead time. If a channel had opened to the 28 pS level and then closed, the expected number of observed openings to the 20 pS level occurring within twice the system dead time (140 μ s) per observed 28 pS opening can be approximated by multiplying the 20 pS channel opening rate time twice the minimum recordable closed duration. The minimum recordable closed duration would be the minimum number of sample points longer than twice the dead time and is 150 μ s for a sampling interval of 50 μ s. The expected percentage of apparent main- to sub-conductance level transitions for all openings would be about 0.026% for 2 μ M-GABA. Since the frequency of main-conductance level openings was higher, the expected percentages of apparent sub-conductance to main-conductance level transitions would be higher, about 0.13%. This estimate for 20 to 28 pS transitions was almost a magnitude too low compared to the observed rate (Table 1) and suggests a correlation between 20 and 28 pS openings.

If the conductance level transitions were direct transitions of a single receptor channel, the principle of microscopic reversibility would predict an equal number of transitions from the main-conductance level to the sub-conductance level and vice versa. Since the number of conductance level transitions from the 28 pS to the 20 pS level and vice versa were approximately equal for openings evoked by GABA alone or in the presence of the steroids, these findings suggested that these observed transitions were likely from channels capable of switching conductance from the 28 pS to the 20 pS levels and vice versa. However, this did not provide evidence that all openings of the 28 and 20 pS conductance levels were from the same set of receptor channels. It does suggest, however, that in some of the receptor channels, the channel may transit from one conductance level to another. If openings to the two conductance levels were from the same set of receptor channels, this transition of conductance levels appears to also confer a change in the gating properties of the receptor channel since the open- and closed-duration time constants and relative areas were different from the two conductance levels. The basis for the conductance level transitions is unknown. The mechanisms may involve minor changes in charge distributions within the channel or perhaps shifts in the configuration of the receptor subunits. Pore charge distribution and receptor subunit composition have been demonstrated to affect conductance of the nicotinic acetylcholine (nACh) receptor (Mishina, Imoto, Noda, Takahashi, Numa, Methfessel & Sakmann, 1986; Imoto, Busch, Sakmann, Mishina, Konno, Nakai, Bujo, Mori, Fukada & Numa, 1988). Multiple conductance levels have not been readily observed for the nACh receptor and direct comparison to the GABA receptor cannot be made. Whatever the process, prolonged periods of activity of one conductance level were observed, indicating that the transitions can be relatively stable. Due to the predominance of the 28 pS level, this conductance certainly has the more stable configuration for the GABA receptor. Even though steroids are highly lipophilic, they do not appear to affect the process regulating the opening of channels to different conductance levels, but rather the process regulating the gating of channels.

Single-channel kinetic properties

Multiple kinetic open states of the GABA receptor are known to exist (Macdonald *et al.* 1989a; Weiss & Magleby, 1989). The increased average single-channel currents in the presence of AND and PRE were due to an increase in time spent in open states. An increase in average channel open duration in the presence of PRE has been reported (Callachan *et al.* 1987; Mistry & Cottrell, 1990) and was consistent with the findings of this study. However, except for the highest AND concentration (see below), the steroids did not increase the open state time constants of the GABA receptor channel, but rather, increased the probability of occurrence of the open kinetic state with the longest time constant and reduced the probability of occurrence of the two states with shorter time constants. This suggests that the steroids did not alter the open states of the GABA receptor channel, but rather, produced an allosteric regulation of the proportion of longer open states evoked by GABA. This mechanism of prolongation of average channel open duration was not like that found for GABA concentration-dependent changes in average open duration. Using similar analysis methods, increased GABA concentration increased

the probability of occurrence of the two open states with longer time constants but without changes in the ratio of the relative frequency of occurrence of the two states (Macdonald *et al.* 1989*a*). Barbiturates also increased the average open duration of the GABA receptor channel (Study & Barker, 1981), and their single-channel kinetic mechanism for prolongation of open duration was similar to that of the steroids (Twyman, Rogers & Macdonald, 1989*a*; Macdonald, Rogers & Twyman, 1989*b*). In mouse spinal neurones in culture, pentobarbitone and phenobarbitone did not increase average main-conductance level open duration by altering time constants of the open states, but by increasing the relative probability of occurrence of the open state with longest time constant (Macdonald *et al.* 1989*b*). Thus, the mechanism for increased average open duration of the GABA receptor channel appears to be similar for the steroids AND and PRE and for the barbiturates, pentobarbitone and phenobarbitone. This mechanism was different than that described for the benzodiazepine, diazepam. Diazepam increased single-channel GABA receptor current primarily by increasing GABA-evoked opening frequency without significant alteration of average channel open or burst durations and without alteration of open- or burst-duration time constants (Study & Barker, 1981; Vicini, Mienville & Costa, 1987; Twyman, Rogers & Macdonald, 1989*b*; Macdonald *et al.* 1989*b*). The results presented here provide physiological evidence that both steroids and barbiturates functionally increase GABA receptor channel average open durations by a similar mechanism. AND and PRE primarily increased the proportion of bursts of openings with the longest open-duration time constant and did not greatly alter burst-duration time constants. This suggested that these steroids primarily affected a transition rate regulating entry to a kinetic state that produced bursts of long-duration openings without altering the number of openings in the burst. This was quite similar to the mechanism for the regulation of bursts by barbiturate and provides further evidence that these steroids and barbiturates prolong GABA receptor channel average open duration and burst duration by a common effector mechanism.

Since the number of active channels recorded at equilibrium was unknown, the increased channel opening frequency in the presence of the steroids could have been due to recruitment of inactive receptor channels. However, opening frequency in runs of single openings was increased in the presence of the steroids compared to GABA alone. This suggests that the steroids were not recruiting additional GABA receptor channels and that the overall increased opening frequency in the presence of the steroids may have been due to an increase in GABA receptor affinity or an alteration in the process of receptor desensitization. The increased opening frequency was more prominent at the higher concentrations of the steroid, and in combination with the increase in average channel open duration, was an effective mechanism for single-channel current enhancement by these steroids.

Multiple closed-duration time constants were found and suggests the presence of multiple kinetic closed states. Single-channel kinetic models of GABA receptor channel gating have contained multiple distinct kinetic closed states (Macdonald *et al.* 1989*a*; Weiss & Magleby, 1989; Twyman *et al.* 1990). The resultant closed-duration time constants from multiple adjacent closed states are a complex combination of the exit rates from the adjacent states (Colquhoun & Hawkes, 1982).

Resolution of closed-duration time constants in the closed-duration frequency histograms is dependent upon the relative weighting of the closed states, the degree of separation of the time constants and the number of closed durations in the frequency distribution. Attributing a specific kinetic mechanism to changes in the long closed-duration time constants would be difficult without testing models by computer simulation. Generally, five exponential components could be resolved well and comprised over 95% of the area of the closed-duration frequency histograms. The increased proportion of area of the components with the three shortest time constants in the closed-duration distributions was consistent with the observed increase in opening frequency. At the higher steroid concentrations, the time constant for component 5 was decreased compared to GABA (Fig. 8). The basis for this decrease was unclear, but possibly was related to altered GABA binding. The steroids have been shown to enhance [³H]muscimol binding (Simmonds *et al.* 1984). Kinetically, facilitation of GABA binding could reduce time spent in a long-lived unbound, closed state or could cause recruitment of previously unactivated receptors. Another possibility is that reduction of a long-lived desensitized state also could reduce a long closed-duration time constant. The decline in macroscopic GABA receptor current during constant application of GABA has been attributed primarily to receptor desensitization rather than chloride shifts (Akaike, Inomata & Tokutomi, 1987). The decline in opening frequency with constant GABA application rather than a change in channel conductance found in this study corroborates this. The effect of steroids on GABA receptor desensitization is unclear although the slower decline in opening frequency in the presence of the steroids seems to implicate an alteration of GABA receptor desensitization.

GABA receptor channel kinetic properties at the highest steroid concentration

The steroid concentration-dependent prolongation of the GABA receptor main-conductance level average open durations was decreased at the 10 μM steroid concentration. This was most apparent for AND. The open-duration time constant for 10 μM -AND also was decreased compared to GABA and the other steroid concentrations and since the relative proportion of this time constant was great, the average open duration was decreased. As a mechanism for the decreased time constant, it is possible that AND was producing open channel block. Notable is the finding that only the longest open-duration time constant was affected at this concentration. The single-channel kinetic basis for an open-channel block mechanism can be explained in the following manner. The dwell time in an open state is inversely related to the sum of the exit rates out of the open state (Colquhoun & Hawkes, 1982). Thus, the open state with the shortest time constant would have the greatest closing rate and the open state with the longest time constant would have the smallest closing rate. Given the presence of three kinetically distinguishable open states, an open-channel blocker may occlude the channel by associating with the pore with equal association rate constants for each open state. If this were the case, an increased concentration of blocker of the open state with the longest open time constant (smallest closing rate) would have a greater relative effect on the total exit rate (sum of the closing rate and the blocking rate) from the open state. Thus, compared to the shorter open state, the longest open state would be the most

sensitive to the presence of an open channel blocker, and its time constant would decrease more significantly with increased concentration of blocker. Small changes in the other open time constants were not distinguished in the present data.

If the steroid produced simple open channel block, the burst-duration time constant resulting from bursts which included openings from the longest open state would be prolonged due to repeated oscillations between the open state and the blocked closed state. The average burst duration and the time constant of component 3 of the burst-duration distributions actually decreased in $10 \mu\text{M}$ -AND (Fig. 11). However, this does not prove that channel block did not occur. A block mechanism by which the steroid molecule remains 'trapped' when the channel is closed can also produce an apparent reduction in the 'measured' burst duration. Since burst durations were 'measured' or analytically identified by their separation of closed durations longer than 5 ms, trapping the steroid molecule for longer than 5 ms can produce a reduction in the average burst durations. The same would be true for a simple open-channel block mechanism whereupon dissociation of the blocker was slow and had a blocked state dwell time longer than 5 ms. The decreased time constant also can be explained by an allosteric mechanism regulating a pre-existing exit rate from the longest open state. This mechanism also may reduce the open-state time constant. Determination of which of these mechanisms was responsible for the reduction of the longest time constant would require more detailed analysis (Colquhoun & Hawkes, 1982; Twyman *et al.* 1991), and therefore, a definite conclusion cannot be reached at the present time. Biphasic concentration response curves for the facilitation of GABA chloride currents have been observed (Morrow, Suzdak & Paul, 1987) and channel blockade at the highest steroid concentrations would be the simplest and most probable explanation for this.

Steroid reduction of the longest open-duration time constant was not observed for the sub-conductance level. This could indicate that the binding pocket for a blocker was different for the 20 and 28 pS conductance levels. The differential effect of an open-channel blocker on a channel with multiple conductances is unknown.

Multiple steroid binding sites

The effect of increasing the relative contribution of the longest open state in the open-duration frequency histogram was still present at the highest steroid concentrations. This suggests that the allosteric regulatory mechanism that promotes the entry into the longest open state was still effective at $10 \mu\text{M}$ -AND. It also suggests that this kinetic mechanism was different to that which produced a decrease in the longest open-state time constant and also implicates the presence of a second steroid binding site.

At high concentrations (greater than $1 \mu\text{M}$), some neurosteroids directly activated the GABA receptor, and the directly activated current was blocked by the GABA receptor antagonist bicuculline, suggesting that the steroids can bind and interact directly with the gating mechanism that opens the chloride channel (Callachan *et al.* 1987; Cottrell *et al.* 1987). The single-channel kinetics underlying direct steroid activation and whether or not the channel is activated by steroids in the same manner as by GABA are unknown. The combined effects of steroid facilitation of the GABA receptor currents and direct steroid activation of the channel on the single-channel kinetics also is unknown.

Multiple steroid binding sites on the GABA receptor have been proposed (Majewska *et al.* 1988; Puia, Santi, Vicini, Pritchett, Purdy, Paul, Seeburg & Costa, 1990). A role for at least one of the GABA receptor subunits (β) has been implicated as a binding site for allopregnanolone and alltetrahydrodeoxycorticosterone. Facilitation of GABA-activated currents and direct activation of bicuculline-sensitive chloride currents by these steroids have been recorded from transfected human β_1 , $\alpha_1\beta_1$, and $\alpha_1\beta_1\gamma_2$ combinations of subunits (Puia *et al.* 1990). Noise analysis revealed that the estimated channel-open durations of steroid enhanced GABA-activated currents from these transfected cells were not prolonged compared to GABA. This was in contrast to the noise analysis in cultured rat spinal neurone results showing increased channel-open durations in the presence of alphaxalone (Barker *et al.* 1987). The stoichiometry of native GABA receptors has not been determined and the basis for the observed differences remains unknown. None the less, it is apparent that neurosteroids can interact with the GABA receptor via different mechanisms including allosteric regulation, direct activation and probably channel block. It remains unclear whether or not each of these actions result from separate binding sites.

Comparison of steroid and barbiturate mechanisms of action at the GABA receptor

This study has extended findings that steroids can potently enhance GABA receptor currents, and analysis of single-channel properties has suggested that the mechanism of prolongation of GABA receptor channel average open and burst durations was 'barbiturate-like' as suggested by earlier reports. However, contrary to that described for barbiturates (Study & Barker, 1981; Macdonald *et al.* 1989*b*; Twyman *et al.* 1989*b*), the steroids increased GABA receptor channel-opening frequency. This differential effect on opening frequency could be accounted for by different binding sites on the GABA receptor. Although steroid and barbiturate binding to the GABA receptor may not be the same, detailed analysis of single-channel gating kinetics revealed that steroids altered gating of the GABA receptor channel in a fashion similar to that of barbiturates. To relate this functional alteration to the GABA receptor structure, AND and PRE specifically stabilized a bursting state of the receptor that was composed primarily of long open states. This similarity in modulation of single channel gating kinetics suggests that both these steroids and the barbiturates may regulate the GABA receptor protein through at least one common effector mechanism.

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