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KINETIC PROPERTIES OF THE $GABA_A$ RECEPTOR MAIN CONDUCTANCE STATE OF MOUSE SPINAL CORD NEURONES IN CULTURE

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SUMMARY

1. The kinetic properties of the main conductance state of γ-aminobutyric $acid_A$ (GABA) receptor channels from somata of mouse spinal cord neurones in cell culture were investigated using patch clamp techniques.

2. Whole-cell GABA receptor currents increased in a concentration-dependent manner from 0·5 to 5 μM.

3. Single-channel currents were recorded with a main conductance state of 27·2 pS and a less frequent conductance state of 15·9 pS. The main conductance state opened singly and in bursts of several openings.

4. Mean open times of GABA receptor main conductance currents were increased and open-time frequency histograms were shifted to longer times as GABA concentration was increased from 0·5 to 5 μM. Three exponential functions were required to fit the histograms at all GABA concentrations, suggesting that the channel opened into at least three open states (O_1, O_2 and O_3). The three functions had the same time constants (1·0 $\pm$ 0·2, 3·7 $\pm$ 0·4 and 11·3 $\pm$ 0·5 ms; mean $\pm$ S.D.) at each concentration. The increase in long open times with concentration was due to a shift in relative frequency of occurrence of openings from the shortest (O_1) to the two longest (O_2 and O_3) open states.

5. Closed-time distributions of closures between main conductance state openings were fitted with multiple exponential functions, suggesting that the channel had several closed states. The two shortest time constants (0·24 $\pm$ 0·03 and 2·0 $\pm$ 0·3 ms) were concentration independent (0·5 to 5 μM). Three longer time constants decreased as concentration increased.

6. Bursts were defined as groups of openings surrounded by closures greater than a critical closed time (t_c = 5 ms). Mean burst durations were increased and burst-duration frequency histograms were shifted to longer times as GABA concentration was increased from 0·5 to 5 μM. Burst-duration frequency histograms were best fitted with three exponential functions. The time constants were concentration independent and were 1·0 $\pm$ 0·2, 5·5 $\pm$ 0·2 and 29·8 $\pm$ 1·6 ms. The increase in burst duration with concentration was due to a relative shift from short duration bursts to longer duration bursts.

7. The shortest burst time constant was similar to the shortest open time constant

suggesting that there was a population of single openings of short duration. The two longest burst time constants were longer than the two longest open time constants, suggesting that the bursts from the two longest burst components were composed of two or more openings.

8. From these data we conclude that the main conductance of the GABA receptor channel has at least three open and two closed states whose time constants are concentration independent. The channel opens into a single-opening burst and into two complex bursts consisting of multiple openings. Single-opening bursts may be composed primarily of openings to the O_1 state while complex bursts may be composed primarily of openings to the O_2 and O_3 states.

9. We present a preliminary model for the gating of the main conductance of the GABA receptor channel.

INTRODUCTION

GABA binds to $GABA_A$ receptors and opens chloride ion channels. Single-channel current recording techniques (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) have shown that single-channel GABA receptor currents are complex (Hamill, Bormann & Sakmann, 1983), with four different current amplitudes corresponding to channel conductances of 44, 30, 19 and 12 pS (Bormann, Hamill & Sakmann, 1987). The most frequent or main conductance state, however, was the 30 pS conductance state. Also, the openings of chloride channels at all conductance states occurred in 'bursts', consisting of a series of openings and short closings.

We have used the patch clamp technique to determine the temporal characteristics of the main conductance state of single-channel GABA receptor currents from mouse spinal cord neurones grown in cell culture. The main state of the GABA receptor chloride channel was studied in outside-out patches exposed to concentrations of GABA from 0·5 to 5 μM.

METHODS

Cell culture

Spinal cords were dissected from 12- to 14-day-old murine fetuses. They were then mechanically dissociated to yield a single-cell suspension and grown in culture medium as described previously (Ransom, Neale, Henkart, Bullock & Nelson, 1977). 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 2 ml extracellular solution which consisted of the following, in mM: 142 NaCl, 8·09 KCl, 1 $CaCl_2$, 6 $MgCl_2$, 10 glucose, 10 Na-HEPES, (pH 7·4). The specific glycine receptor antagonist, strychnine (200 nM; Sigma), was added to the extracellular solution to ensure that no glycine receptor currents were included in the analysis. This concentration of strychnine did not affect GABA responses. Tetrodotoxin (1 μM) was added also to the extracellular solution used for whole-cell recording to eliminate spontaneous action potentials. The solution used in the micropipettes for patch clamp and whole-cell experiments contained, in mM: 153 KCl, 1 $MgCl_2$, 10 Na-HEPES, 5 EGTA, 1 NaOH, 2 KOH (pH ~7·38). This combination of extracellular and patch clamp micropipette solutions resulted in a chloride equilibrium potential (E_{Cl}) of 0 mV and a potassium equilibrium potential (E_K) of −75 mV. All recordings were performed at room temperature (20–23 °C).

GABA and drug 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 0·5, 1, 2 or 5 μM on the day of each experiment. GABA was applied to neurones or patches by pressure ejection from micropipettes. Picrotoxin (Sigma) was dissolved in external bathing solution at a concentration of 10 μM on the day of the experiment. Bicuculline (Sigma) was dissolved in dimethyl sulphoxide (DMSO) to a concentration of 10 mM. Serial dilutions were made in extracellular solution to a final concentration of 0·2 μM with a final DMSO concentration of not more than 0·01 %. Bicuculline was not used after it had been in solution for more than 2 h. A mixture of GABA (2 μM) and picrotoxin (10 μM) or GABA (2 μM) and bicuculline (0·2 μM) was applied to the cell via pressure ejection micropipettes in the presence of a large-tip or diffusion micropipette containing picrotoxin (10 μM) or bicuculline (0·2 μM), respectively. Pressure ejection pulses of 20 s were used for all of the GABA concentration response measures and for 5 s for GABA responses with and without antagonist application. GABA was applied alone for 30 s to evaluate desensitization. Micropipettes were moved to within 50 μM of neurones or patches only during the time of each application.

Current recording

Recording micropipettes were made up to 24 h prior to use from Microhematocrit Capillary tubes (Fisher Scientific, Pittsburgh) and were pulled in two stages using a model 700-D puller (David Kopf Instruments). Micropipettes used for either pressure ejection or for diffusion of GABA or drugs were pulled from 1·2 mm diameter glass. Tips were broken to give tip diameters of 15–25 μm. Micropipettes were backfilled with extracellular solution containing the desired concentration of GABA and/or other drug. A pressure of 4–7 kPa above ambient was used for drug ejection. To avoid dilution in the extracellular solution, the micropipettes containing GABA or drugs were kept out of the extracellular solution whenever the drugs were not being applied. Prior to application, a pressure pulse (1–5 s) was applied distant from the recording site to eject diluted solution from the micropipette tip.

Both whole-cell and excised outside-out patch clamp recordings were obtained using a model L/M EPC-7 amplifier (List Medical Instruments, Darmstadt). For whole-cell recordings, responses were low-pass filtered (3 dB at 600 Hz, 8-pole Butterworth; A.P. Circuit Corporation) and recorded on a chart recorder (Gould Inc.). Patch clamp command potentials and single-channel currents were low-pass filtered (3 dB at 10 kHz, 8-pole Bessel filter) and recorded on a video cassette recording (VCR) system (Sony SL-2700, modified to 0–20 kHz) via a digital audio processor (Sony PCM-501ES, 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).

During whole-cell recordings, neurones were voltage clamped at -75 mV, and membrane conductance was determined from constant short-duration (100 ms) voltage commands applied at 1 s intervals.

Single-channel current analysis

Following excision of outside-out patches, the initial application of GABA often evoked channel openings (80 % of patches). In all responsive patches, however, multiple channel openings were evoked. For the present study, data were accepted for analysis if only rare multiple openings (less than 1–3 % of detected openings and no evidence of three or more simultaneous openings) were detected during that application of GABA (128 patches). If possible, all concentrations of GABA were applied to each patch prior to patch disruption or evidence of desensitization.

For analysis, single-channel data were played back from the VCR system and digitized (8 kHz, 14-bit, 40·96 points/pA, Tecmar A/D converter) 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 to 25 s. Single-channel data were analysed by computer using locally written analysis programs. Channel opening amplitude distributions were found to be described by two Gaussian functions (Colquhoun & Sigworth, 1983). A smaller third and a larger fourth conductance level was observed in the recordings but could not be resolved adequately in the amplitude distributions. Channel openings to three independent amplitudes and their respective closings were detected using the 50 % threshold crossing method. To be accepted as a valid opening, mean

current amplitude during an opening had to be within a specified window (approximately 1·5–2 standard deviations of the noise variance). The output contained condensed data consisting of a series of open and closed durations and their amplitudes.

To obtain an accurate estimation of channel amplitude (Colquhoun & Sigworth, 1983), openings were constrained to be greater than twice the rise time of the low-pass filter (rise time = 340 μs, determined empirically). Single-channel currents of large ($\sim$2·0 pA) and moderate ($\sim$1·2 pA) amplitudes were usually recorded with the larger channel occurring much more frequently. Currents with a smaller amplitude ($\sim$0·8 pA) were inadequately resolved due to poor signal-to-noise ratio, and currents with very large amplitudes ($\sim$3·4 pA) were recorded only rarely.

For temporal analysis, the condensed data were re-analysed for the main conductance state. Openings to the two other conductances and multiple simultaneous openings were rejected. Detected open and closed times less than twice the dead time (dead time = 130 μs) of the system including the low-pass 8-pole Bessel filter (3 dB cut-off at 1 kHz) were counted as unresolved open and closed times, respectively. The open and closed times reported in this study are observed times. Due to the presence of missed or unresolved events, the observed event 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 (open-time correction in Table 1). This type of correction, however, does not correct for the effect of missed closures. Accurate correction of both open- and closed-time distributions cannot be done without a specific model of the gating mechanism, which at present is unavailable (McManus, Blatz & Magleby, 1987).

Durations of idealized openings and closings and of burst durations from all patches were pooled and collated into frequency histograms. Bin widths were 0·33 % of the range of time the histogram covered and were well within the criteria for accurate representation (Blatz & Magleby, 1986). Frequency histograms of open times were fitted from bins starting from at least twice the system dead time. Histograms were fitted to a sum of n exponential functions (f(t); eqn (1)) using a locally modified non-linear curve-fitting routine (NFITS, M. Sloderbeck & C. J. Lingle, Florida State University, Tallahassee, FL, USA):

$$f(t) = \sum_{i=1}^{n} a_i \exp(-t/\tau_i). \quad (1)$$

In eqn (1), a_i and τ_i were the relative area and time constant of the ith exponential component, and $a_1 + a_2 + \ldots a_n = 1$.

Estimates of exponential areas and time constants 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 with increasing numbers of exponentials 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). Distributions of open or closed times were fitted using the same bin widths and over the same range for each concentration of GABA.

Analysis of closed times presented two additional difficulties. Since the data contained a small number of multiple simultaneous main-state openings and openings to other conductance states, it was difficult to determine unambiguously which closed times represented the 'main-state closed times'. For the purposes of this study, closed times were analysed using three different assumptions. First, all closed times between all openings (multiple-, main- and sub-conductance states) were analysed. Second, closed times following main-state openings were analysed. In this analysis, non-main-state and multiple open times and closed times following all non-main-state and multiple openings were rejected (deleted). Third, only closed times between main-state openings were analysed. While there were slight quantitative differences among the results obtained using these three closed-time definitions, there were no qualitative differences. The closed-time histograms presented in this paper were obtained using the third closed-time assumption.

Definition of bursts

Bursts may be defined as openings or groups of openings separated by relatively long closed periods (Colquhoun & Sigworth, 1983). For the purpose of this analysis, a critical closed time, t_c, was chosen such that all openings separated by closures less than t_c belonged within a burst, and bursts were separated by closures greater than t_c. A t_c may be selected to lie between a fast closed time constant, t_f, and a slow closed time constant, t_s. A modification of the equal proportion of

misclassifications method of Colquhoun & Sakmann (1985) was used to select a t_c. A t_c was calculated such that the proportion of misclassified closures belonging to the distribution of fast closures (closings longer than t_c) was equal to the proportion of misclassified closures belonging to the distribution of slow closures. If the system dead time (t_d) was considered in the proportion of actually detected events, t_c can be determined from eqn (2):

$$\frac{1}{\tau_f}\int_{t_c}^{\infty}\exp(-t/\tau_f)\,dt = \frac{1}{\tau_s}\int_{2t_d}^{t_c}\exp(-t/\tau_s)\,dt. \quad (2)$$

The t_c values obtained using the three closed-time definitions were similar. In this study the t_c was determined using the third closed-time definition.

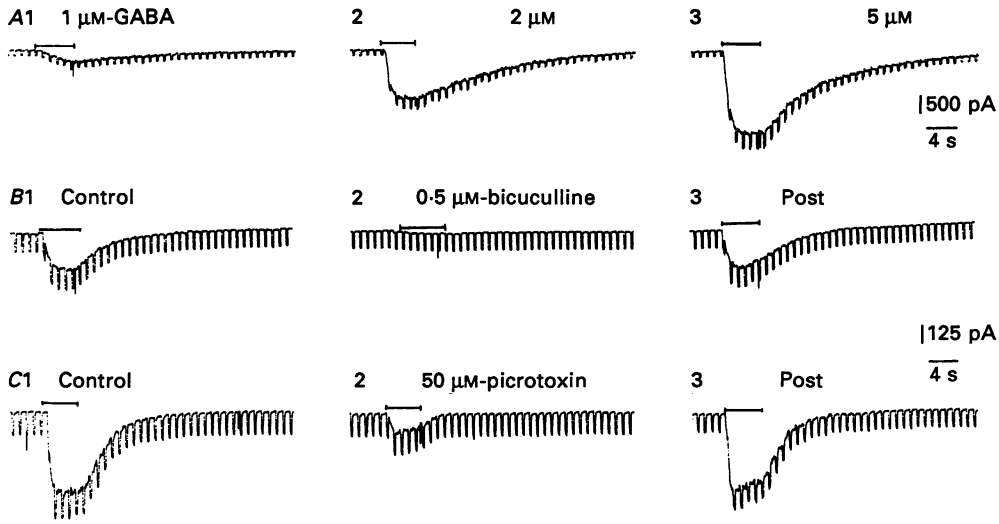

Fig. 1. Whole-cell GABA responses. *A*, GABA (1, 2 and 5 μM) evoked concentration-dependent inward currents and increases in membrane conductance. *B*, GABA (2 μM) evoked an inward current which was inhibited reversibly by bicuculline (0·5 μM). *C*, GABA (2 μM) evoked an inward current which was inhibited reversibly by picrotoxin (50 μM). Neurones were held at −75 mV using the whole-cell patch clamp technique, and the chloride equilibrium potential was 0 mV. GABA was applied for 5 s to the soma of each neurone. Small hyperpolarizing commands (100 ms) were applied at 1 Hz and the small inward currents required for these commands are apparent in the current records.

RESULTS

GABA receptor whole-cell currents

Whole-cell recordings were obtained at −75 mV. GABA was applied by pressure ejection and reproducibly evoked inward currents. The inward currents evoked by GABA were concentration dependent (Fig. 1*A*). At 0·5 μM, minimal current was evoked, but at 1–5 μM, increasing currents were evoked. The GABA-evoked inward currents were reversibly antagonized by the GABA antagonists, bicuculline (0·5 μM; Fig. 1*B*) and picrotoxin (50 μM; Fig. 1*C*).

With short applicatons of GABA (5 s), peak inward current was reached within 1 s, and a relatively stable plateau was achieved. After GABA application, the inward current returned to baseline in 5–15 s. However, with longer (30 s) GABA applications, inward current and the associated increase in membrane conductance

declined slightly over time, possibly due to desensitization (not illustrated). To minimize desensitization and to allow application of several concentrations of GABA, all studies of single-channel currents were performed with short (20 s) GABA applications.

GABA receptor single-channel currents

Excised outside-out patches were obtained from spinal cord neurones. Following excision of the patch at 0 mV, abundant outward potassium currents were commonly recorded. Following patch hyperpolarization to −75 mV (E_K), potassium currents were abolished. Prior to GABA application occasional brief spontaneous currents were recorded (Fig. 2*A*1). Following application of GABA (0·5–5 μM), bursting inward chloride currents were evoked in most patches (80%; Fig. 2*A*2). The bursting currents evoked by GABA were reversibly reduced by bicuculline (0·2 μM; Fig. 2*A*3) and by picrotoxin (10 μM; Fig. 2*A*4). Responses evoked from a single patch were often reproducible and could be evoked for up to 30 min. Occasionally, after repeated application, current activity decreased significantly or stopped for prolonged periods. Therefore, GABA receptor currents were accepted for analysis only if they were stable during the first two or three applications of GABA. Multiple simultaneous openings comprised only 1·7% of all openings.

At least two conductance states were apparent in the single-channel records (Fig. 2*A*2). The larger conductance state (Fig. 2*A*2, **) was recorded more frequently than the smaller conductance state (Fig. 2*A*2, *). Consistent with this observation, current amplitude histograms (0·04 pA bin width) contained a distribution of amplitudes that were best fitted by using two Gaussian functions (not illustrated). Current amplitudes were 2·04±0·26 and 1·19±0·18 pA (mean±S.D.) at −75 mV. Single-channel currents reversed at 0 mV. Channel conductances (mean±S.D.) from all patches ($n = 65$) were 27·2±3·4 and 15·9±2·4 pS for the high- and low-conductance states, respectively. For valid openings, the larger conductance level accounted for 72% of the open duration, and therefore had a larger amplitude in the double-Gaussian distribution. The smaller conductance level accounted for only 28% of the open duration. There was no apparent concentration shift in the relative proportion of the main (range, 60–85% of open duration) and subconductance (range, 15–40% of open duration) states, although analysis of the subconductance state was insufficiently rigorous to completely exclude a shift in relative proportion of openings. The kinetic analyses reported in this study were performed only on the larger or main conductance state.

Current amplitude of the main conductance state varied linearly with membrane potential and inverted near 0 mV (E_{Cl}; Fig. 2*B*). In a representative patch over the range of −80 to +60 mV, the current–voltage relationship of the main conductance state was linear and had a slope conductance of 27 pS.

Open times were concentration dependent

GABA evoked complex currents (Fig. 3*A*1) which were often brief and produced by a single chloride channel opening (Fig. 3*A*2, *) or were prolonged and consisted of sequential single-channel openings and closings (Fig. 3*A*2, bar). With increasing temporal resolution, the pattern of openings was complex and consisted of multiple consecutive openings and closings (Figs 3*A*3 and 4).

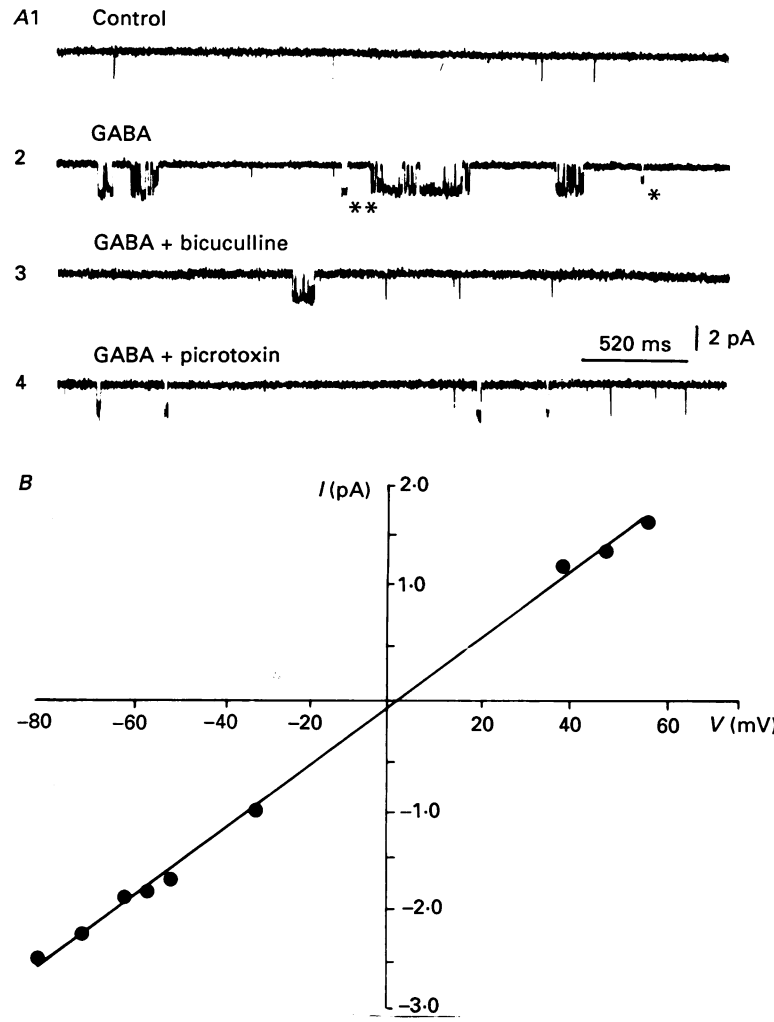

Fig. 2. *A*, GABA (2 μM)-evoked single-channel currents in outside-out patches from spinal cord neurones voltage clamped at −75 mV. Solutions were as described in the text. *A*1, in the absence of applied GABA, brief spontaneous inward unitary currents were recorded. *A*2, GABA (2 μM) evoked increased channel openings that were composed of a frequent high or main conductance state (**) and a rare lower conductance state (*). *A*3, inward currents evoked by GABA (2 μM) were reduced by bicuculline (0·2 μM). *A*4, inward currents evoked by GABA (2 μM) were reduced by picrotoxin (10 μM). Responses returned to control after removal of the antagonist (not illustrated). *B*, single-channel current–voltage relationship of the main conductance state evoked by GABA (2 μM) in an outside-out patch. Slope conductance was 27 pS. Signal-to-noise ratio of channel openings was too low for reliable channel amplitude measurement when the membrane potential was ±40 mV.

The currents evoked by GABA were concentration dependent. Prior to the application of GABA, rare, brief spontaneously occurring currents were seen (Fig. 3*B*1). With increasing concentration, GABA evoked an increasing frequency of openings and bursts with increasing complexity (Fig. 3*B*2–5). Multiple 20 s recordings of chloride currents were summed and averaged to produce an ensemble average

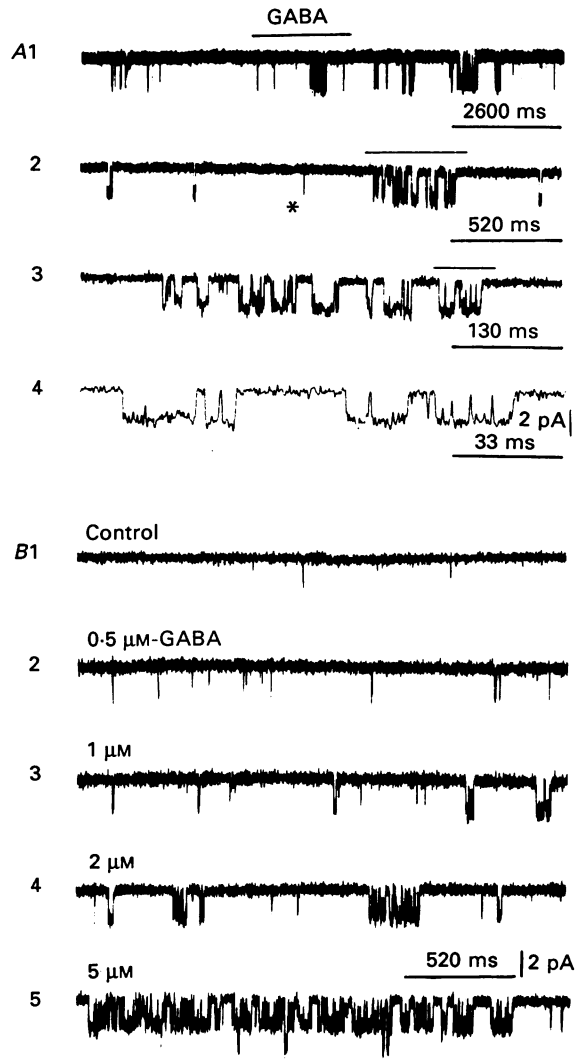

Fig. 3. *A*, GABA (2 μM)-evoked inward currents contained single and bursting currents. Currents are shown at increasing time resolution (*A*1–4). The portion of current record under the bar above each trace is presented expanded in time in the trace below it. The asterisk (*) indicates a brief, solitary opening. Time calibration for each trace is shown on the right below the trace. Current calibration at lower right applies throughout. *B*, concentration dependence of single-channel currents evoked by GABA (0·5, 1, 2 and 5 μM). Prior to application of GABA, patches frequently had rare, brief channel openings (*B*1). Opening frequency increased with increasing concentration (*B*2–5).

current per application. To assess total charge transfer, multiple simultaneous openings and other conductance states were not rejected. Average current per GABA application was concentration dependent. In the absence of applied GABA, control recordings contained rare, brief openings. When GABA was applied, this resulted in an average current per application of 0·58 pA by 0·5 μM (sixty-four patches; 165 GABA applications), 1·53 pA by 1 μM (fifty patches; 131 GABA applications), 1·93

pA by 2 μM (eighty-three patches; 193 GABA applications) and 2·30 pA by 5 μM (fifty patches; eighty-one GABA applications) GABA. The time course of the ensemble-averaged GABA (2 μM)-evoked single-channel current was similar to the GABA (2 μM)-evoked whole-cell current (not illustrated).

From 0·5 to 2 μM the mean frequency of channel opening increased from 2·0 to 8·5 openings per second (Table 1). At 5 μM, opening frequency was 6·2 per second. Mean open times of GABA receptor currents were concentration dependent (0·5–5 μM),

TABLE 1. GABA receptor main conductance state open and closed properties

GABA concentration (μM)	0·5	1·0	2·0	5·0
Openings/s	2·0	3·7	8·5	6·2
Mean open time (ms)	4·0	4·5	5·3	6·0
Corrected mean open time (ms)	3·7	4·4	5·0	5·7
Mean closed time (ms)	185·3	97·2	41·0	27·9
Percentage open	0·8	1·7	4·5	3·7
Number of openings	6628	12551	24251	8348
Number of patches	64	50	83	50
Number of GABA applications	165	131	193	87

Openings per second, mean open time, mean closed time, percentage open and number of openings were derived from detected openings (see Methods). Mean closed time refers to the mean closed duration between main conductance state openings. Corrected mean open time was calculated by taking the sum of the relative area (a) of each exponential component in the open-time histogram multiplied by the time constant (τ) of the component (corrected mean open time $= (a_1\tau_1 + a_2\tau_2 + a_3\tau_3)/(a_1 + a_2 + a_3)$).

increasing from 4·0 to 6·0 ms (Table 1). When corrected for undetected openings, mean open time increased from 3·7 to 5·7 ms. As a result of increased opening frequency and mean open time, the percentage of time the channel was open in the main conductance state increased from 0·8 to 4·5% for 0·5–2 μM-GABA. It decreased slightly in 5 μM-GABA to 3·7%.

The distributions of open times were fitted by multiple exponential functions

The distributions of open times produced by the application of GABA (0·5–5 μM) were obtained by pooling open times from several patches at each concentration. Distributions of open times changed with increasing GABA concentration from 0·5 to 5 μM (Fig. 4). As GABA concentration increased, there was a shift in the open-time distribution to longer open times due to an increase in the relative frequency of occurrence of longer openings.

Open-time frequency histograms were fitted to the sums of exponential functions. At all concentrations, three exponential functions were required to best fit the open-time distributions (Fig. 4). The exponential functions with the shortest, middle and longest time constants were called components 1, 2 and 3, respectively. The time constants for components 1, 2 and 3 were concentration independent for GABA applications of 0·5, 1, 2 and 5 μM (Fig. 5*A*). Over the GABA concentration range of 0·5–5 μM ($n = 4$ concentrations), the component 1 time constant ranged from 0·7 to 1·2 ms with a mean of $1·0 \pm 0·2$ ms (mean $\pm$ S.D.). The component 2 time constant ranged from 3·2 to 4·1 ms with a mean of $3·7 \pm 0·4$ ms. The component 3 time

constant ranged from 10·7 to 11·8 ms with a mean of 11·3 ± 0·5 ms. The relative area of each component was a measure of the relative frequency of occurrence of each component opening. The relative area of the open-time distribution represented by component 1 decreased with increasing GABA concentration while the relative areas of components 2 and 3 increased as a function of concentration (Fig. 5*B*). Thus, the GABA concentration-dependent increase in mean open time was due to a concentration-dependent shift in the relative frequency of occurrence of the three components with no alteration in their time constants.

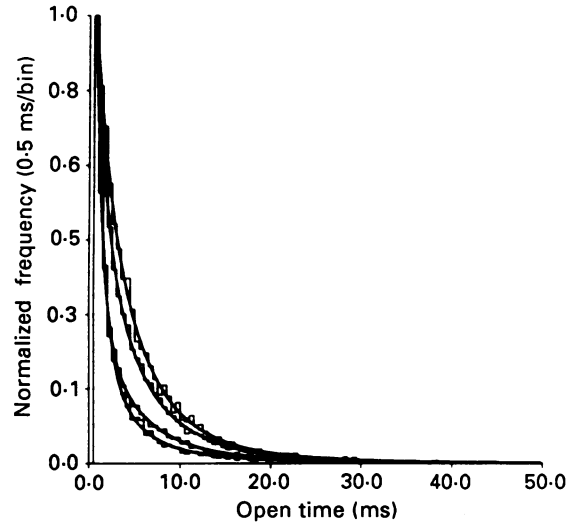

Fig. 4. GABA open-time frequency histograms were concentration dependent and fitted with three exponential functions. Frequency distributions of openings evoked by GABA (0·5, 1, 2 and 5 μM) were put into 0·5 ms bins and displayed over a range of 1–50 ms. Distributions were normalized and overlayed to display relative frequency distribution. Histograms were fitted with three exponential functions and curves were drawn according to the fits (see text). The lowest curve is for 0·5 μM-GABA and the next to lowest curve is for 1 μM. The next-to-highest curve is for 2 μM-GABA and the highest curve is for 5 μM.

Closed times were not all concentration dependent

The distribution of closed times ranged from the shortest accurately detected durations of 375 μs to several seconds. The mean closed times between sequential main conductance level openings were concentration dependent, decreasing from 186 to 28 ms as GABA concentration was increased from 0·5 to 5 μM GABA (Table 1).

Due to the wide range of closed times, closed-time distributions were obtained with two different bin magnitudes. The distribution of short closed times (Fig. 6*A*) ranged from 0·375 to 37·5 ms in bins of 0·125 ms. Long closed times (Fig. 6*B*) ranged from 25·0 to 7500 ms in bins of 25·0 ms. The short closed times varied little as a function of concentration from 0·5 to 5 μM (Fig. 6*A*), while the longer closed times decreased with increasing GABA concentration (Fig. 6*B*).

Closed-time frequency histograms were best fitted by multiple exponential functions

Multiple exponential functions were required to fit the closed-time frequency histograms. The best fits for three exponential functions are shown superimposed on the short and long closed-time frequency histograms (Fig. 6). It is apparent that the closed times for all concentrations were similar for short closed times but differed substantially for long closed times. Thus, it appeared likely that there were

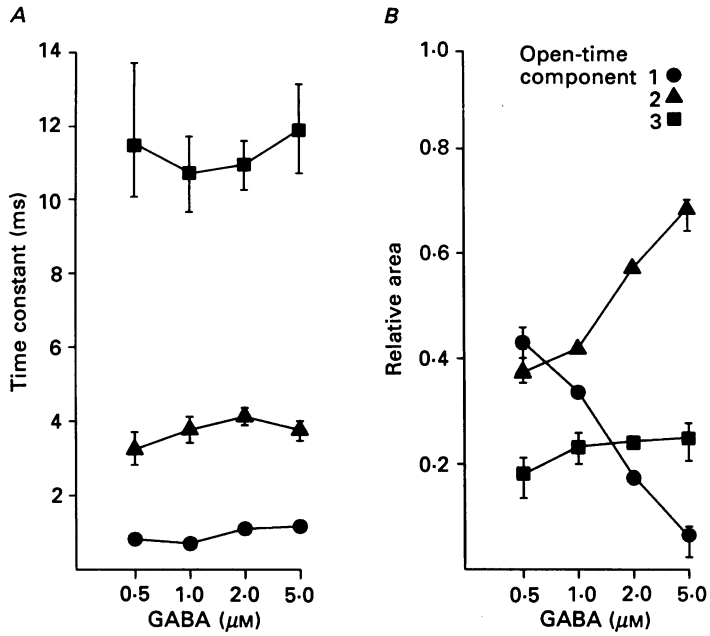

Fig. 5. Concentration dependence of time constants and relative area of open-time frequency histogram curve fits. *A*, curves were fitted with three exponential functions for each concentration of GABA (see Fig. 4 for plots). Time constants of all three components were concentration independent. Components 1, 2 and 3 correspond to the exponential functions with the shortest, intermediate and longest time constants, respectively. Error bars represent likelihood intervals ($m = 2$). Ranges smaller than the symbol were not shown. *B*, the relative area of component 1 decreased with increasing concentration but the relative areas of components 2 and 3 increased.

concentration-dependent long closed times (Fig. 7*B*) with relatively concentration-independent short closed times (Fig. 7*A*). When averaged across the four concentrations, these two components (1 and 2) had mean time constants of $0{\cdot}24 \pm 0{\cdot}03$ and $2{\cdot}0 \pm 0{\cdot}3$ ms. Since the closed-time exponential components were not obtained by simultaneous fitting, the relative areas of the exponential functions could not be obtained reliably.

Mean burst durations and burst frequencies were concentration dependent

Bursts were defined as openings separated by closings greater than t_c. A value for t_c was calculated using a modification of the equal proportion of misclassifications method of Colquhoun & Sakmann (1985) (see Methods). Since the shortest two closed

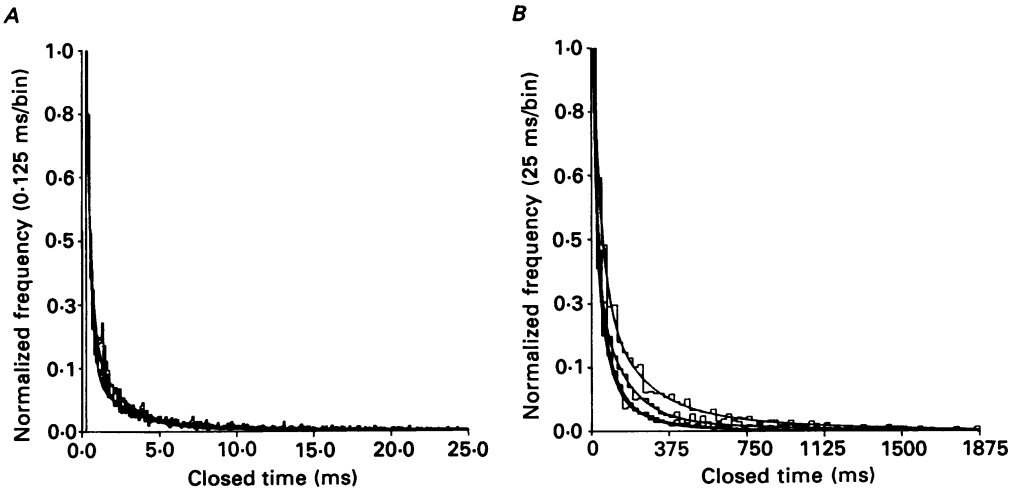

Fig. 6. Closed-time frequency histograms over two different ranges were fitted with multiple exponentials. Distributions from GABA (0·5, 1, 2 and 5 μM) were normalized and overlayed. Histograms were fitted with three exponentials (see text). *A*, short closed times were put into 0·125 ms bins and displayed over a range of 0·375–25 ms. Distributions varied little over these concentrations. *B*, long closed times were put into 25·0 ms bins and displayed over a range of 25–1875 ms. Closed times were longer in the lower concentrations.

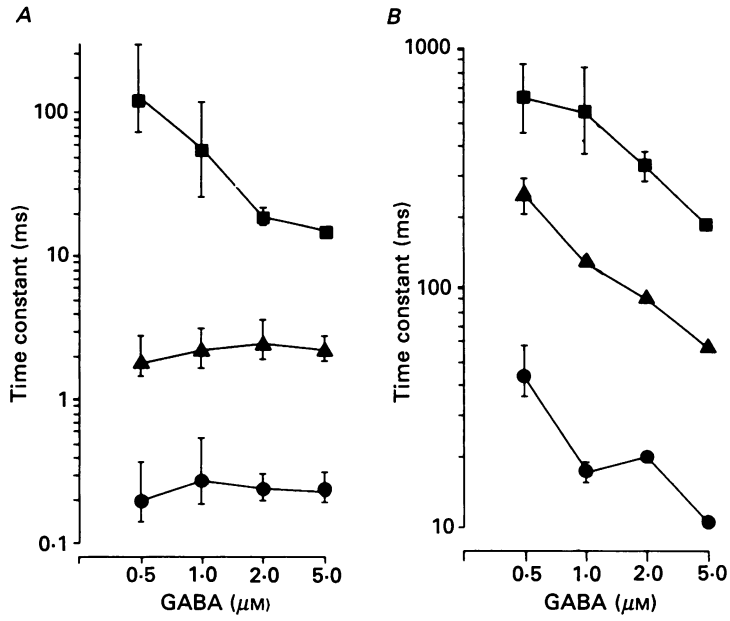

Fig. 7. Concentration dependence of time constants of closed times. Frequency histograms were fitted with sums of exponential functions (see Fig. 6 for plots). The number of significant exponential functions was determined by χ^2 analysis. Components 1–3 corresponded to the exponentials with the shortest to the longest time constants in each histogram distribution, respectively. Error bars represent likelihood intervals ($m = 2$). Ranges smaller than the symbol were not shown.

time constants were concentration independent, t_c was determined between the second and the third time constants in the closed-time distributions for each concentration. Solving eqn (2) (Methods section) resulted in a mean t_c of 5 ms.

As GABA concentration was increased, channel openings occurred more frequently in bursts (Fig. 3*B*). As GABA concentration was increased from 0·5 to 2 μM, the frequency of bursts increased from 1·3 to 4·0 bursts per second, and the mean interburst close time decreased from 660 to 196 ms (Table 2). In 5 μM-GABA, burst frequency was 2·5 per second and mean interburst closed time was 286 ms.

TABLE 2. GABA receptor main conductance state burst properties

GABA concentration (μM)	0·5	1·0	2·0	5·0
Bursts/s	1·3	2·2	4·0	2·5
Mean interburst closed time (ms)	660·0	366·0	196·0	286·0
Mean burst duration (ms)	7·2	8·7	12·9	17·1
Corrected mean burst duration (ms)	7·4	8·9	12·2	15·2
Percentage of analysis time in burst	2·4	4·5	11·1	14·8
Mean percentage intraburst open time	88·4	88·5	88·1	88·2
Number of bursts	4995	10750	13964	4561

Bursts/s, mean interburst closed time, mean burst duration, percentage of analysis time in burst, mean percentage intraburst open time and number of bursts were derived from detected bursts and openings and closings within bursts (see Methods). Bursts were separated by closures greater than 5·0 ms. Mean interburst closed time refers to the mean closed duration between bursts containing main conductance state openings. Percentage intraburst open time was calculated by taking the percentage of the total open time in a burst divided by the total time in a burst (total open time plus total intraburst closed time). Corrected mean burst duration was calculated by taking the sum of the relative area (a) of each exponential component in the burst duration histogram multiplied by the time constant (τ) of the component (corrected mean burst duration = $(a_1\tau_1+a_2\tau_2+a_3\tau_3)/(a_1+a_2+a_3)$).

Burst durations of GABA receptor currents were concentration dependent from 0·5 to 5 μM. Mean burst increased from 7·2 to 17·1 ms (Table 2). The percentage of analysis time spent in a burst increased from 2·4 to 14·8%. In contrast to these burst properties, the percentage of open time within a burst (percentage intraburst open time) was concentration independent, varying little from 88·1 to 88·5%.

Burst-duration frequency histograms were best fitted by multiple exponential functions

Burst-duration frequency histograms were obtained by pooling burst durations from several patches at each concentration. The burst-duration frequency histograms changed with increasing GABA concentration from 0·5 to 5 μM (Fig. 8). As GABA concentration increased, there was a shift in the burst-duration distribution to longer durations, consistent with an increase in the relative frequency of occurrence of longer bursts.

To determine the basis for the increased relative frequency of occurrence of longer bursts by increasing concentrations of GABA, the burst-duration distributions were fitted to the sums of exponential functions. The burst-duration frequency histograms for all three GABA concentrations were best fitted with three exponential functions. Components 1–3 represented bursts from the shortest to the longest durations (Fig. 9). The time constants of the components did not vary with GABA concentration (Fig. 9*A*). Component 1 ranged from 0·8 to 1·2 ms with a mean of

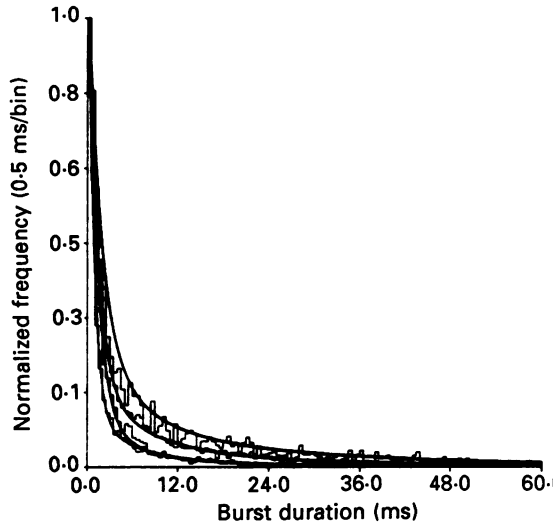

Fig. 8. Burst-duration frequency histograms were concentration dependent and fitted by multiple exponential functions. GABA (0·5, 1, 2 and 5 μM) burst durations were put into 1·0 ms bins and displayed over a range of 1·0–60 ms. There was a concentration-dependent shift in burst durations to the right. Histograms were fitted with three exponential functions (see text).

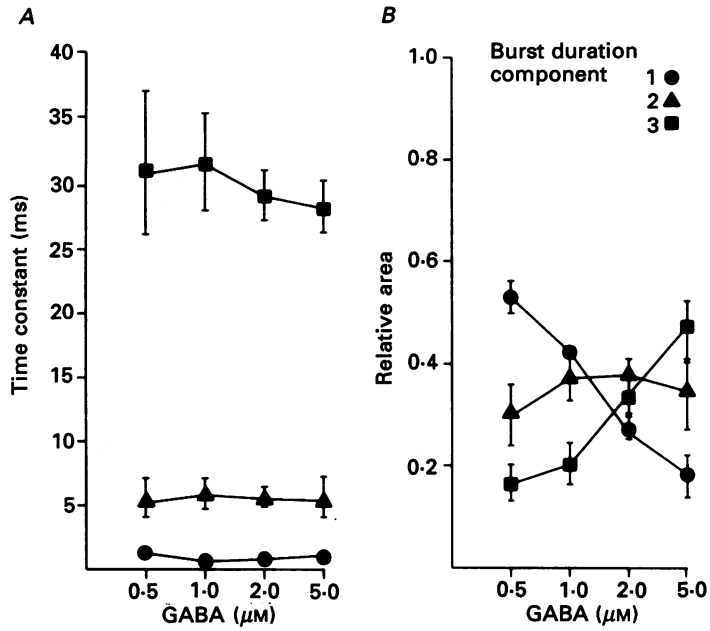

Fig. 9. Burst-duration frequency histograms were fitted with three exponential functions for each concentration of GABA (0·5, 1, 2 and 5 μM) (see Fig. 8 for plots). Components 1–3 corresponded to the exponential functions with the shortest to the longest time constants, respectively. Error bars represent likelihood intervals ($m = 2$). Ranges smaller than the symbol were not shown. *A*, the time constants for the components were concentration independent. *B*, the relative area of component 1 decreased with increased GABA concentration but the relative areas of components 2 and 3 increased.

1·0 ± 0·2 ms; component 2 ranged from 5·2 to 5·7 ms with a mean of 5·5 ± 0·2 ms; component 3 ranged from 28·9 to 31·4 ms with a mean of 29·8 ± 1·6 ms. As GABA concentration increased, the relative proportion of component 1 bursts was reduced, and the relative proportion of component 2 and 3 bursts was increased (Fig. 9*B*). Thus, three types of bursts with three different mean durations were evoked by GABA. Increasing GABA concentration resulted in a shift in the burst-duration frequency histogram to longer durations by reducing the relative proportion of shorter bursts and by increasing the relative proportion of longer bursts.

DISCUSSION

Conductance

We have recorded GABA receptor single-channel currents from outside-out patches obtained from mouse spinal cord neurones grown in cell culture. At −75 mV, two current amplitudes were present with average conductances of 27·2 and 15·9 pS in symmetrical chloride solutions. The dominant or main conductance state was the 27·2 pS state. Previous reports have identified four conductance states of 44, 30, 19 and 12 pS (Bormann *et al.* 1987); the largest (44 pS) and smallest (12 pS) conductance states occurred only rarely and the main conductance state was 29–30 pS (Sakmann, Hamill & Bormann, 1983; Mathers, 1985; Bormann *et al.* 1987). The conductances of the main and subconductance states were the same when evoked by GABA concentrations from 0·5 to 5 μM. Thus, the increased current produced by increased GABA concentration was due to an increase in the frequency of occurrence of channel opening, a change in the relative occurrence of the main and subconductance state and/or a change in the gating properties of the channel.

Open properties

Jackson, Lecar, Mathers & Barker, (1982), Sakmann, Hamill & Bormann, (1983), Bormann & Clapham (1985), Mathers (1985) and Martin (1985) have provided preliminary descriptions of the kinetic properties of the main conductance state of the GABA receptor channel. They have described a chloride channel activated by GABA that has the following properties: (1) its opening frequency is enhanced by GABA; (2) the openings occur singly or in bursts; (3) open-time distributions are composed of at least two exponential functions; (4) closed-time distributions are composed of at least three exponential functions.

In the present study, GABA receptor channel properties varied with GABA concentration. Opening frequency increased with low agonist concentration similar to that described for the frog (Colquhoun & Sakmann, 1985) rat (Jaramillo & Schuetze, 1988) and mouse (Jackson, 1988) muscle nicotinic cholinergic (ACh_n) receptor channel, the crayfish (Dudel & Franke, 1987) and locust (Kerry, Ramsey, Sansom & Usherwood, 1988) muscle glutamate receptor channel, and the *Ascaris suum* muscle GABA receptor channel (Martin, 1985). While some authors feel that many detected openings are incompletely resolved bursts, the mean open or burst duration has also been found to increase with low concentrations of agonist in the same studies. In the present study mean open duration and mean burst duration increased with GABA concentration.

The frequency distributions of open durations were multiexponential. We described three open-time exponential functions with time constants that were stable over a concentration range of 0·5–5 μM-GABA. The finding that the three open time constants were concentration independent suggests that the GABA receptor channel may have at least three open states. It is possible that additional open states with shorter mean dwell times were undetected in this study due to limited frequency resolution. We designated exponential components 1–3 to correspond to open states O_1–O_3, respectively, with state O_1 having the shortest and state O_3 the longest mean open time. The increase in current produced by increasing GABA concentration was achieved, at least in part, by an increase in the frequency of channel opening, and a shift in the relative proportion of less stable, brief openings (state O_1) to more stable, longer openings (states O_2 and O_3).

We tried to limit the analysis to records containing rare simultaneous multiple openings to decrease the likelihood of including multiple receptor patches. The increase in current for 5 μM-GABA may have resulted from an increase in recruitment of channels in the patch in addition to changes in the kinetic properties of a single channel (Erickson, Goldstein, Holowka & Baird, 1987). If too frequent simultaneous openings occurred, patches were rejected from our analysis. Also, higher concentrations of GABA appear to increase desensitization (Akaike, Inomata & Tokutomi, 1987). The decrease in main-conductance channel opening frequency seen at 5 μM-GABA compared to 2 μM may have been due to the limitations of our analysis and/or desensitization. It was noted that the average current per GABA application, which included current from all conductance states and multiple simultaneous openings, increased as concentration increased.

The evidence to date suggests that at least two GABA molecules must be bound for complete activation of the channel (Simmonds, 1981; Nowak, Young & Macdonald, 1982; Bormann & Clapham, 1985; Casalotti, Stephenson & Barnard, 1986). Sakmann *et al.* (1983) also suggested that at least two GABA binding states were present on the receptors since the average duration of intervals between elementary currents within a burst (which provides an estimate of the effective channel opening rate) decreased more than linearly when GABA concentration was increased from 5 to 10 μM.

Whether or not a single bound state can produce channel opening is unclear (Colquhoun & Sakmann, 1981; Cull-Candy, Miledi & Parker, 1981; Colquhoun & Sakmann, 1985). GABA (0·5 μM) evoked openings that were predominantly to state O_1. With increased GABA concentration, the relative frequency of state O_1 openings decreased compared to the frequency of O_2 and O_3 openings. Although further investigation of burst kinetics is necessary, this suggests that a portion of openings to state O_1 or brief openings may represent a singly GABA-liganded open state.

At high GABA concentrations state O_2 and O_3 openings represented 70–80% of openings. Furthermore, the time constants for states O_2 and O_3 were much longer than for state O_1. Thus, the majority of current was through open states O_2 and O_3. The frequency of opening to these stable, long open states increased with concentration, suggesting that these states occurred after GABA binding. Thus, openings to states O_2 and O_3 probably occurred primarily when the GABA receptor was doubly liganded.

Closed properties

GABA receptor channel closed properties also varied with concentration. Mean short closed times varied little but mean longer closed times decreased with concentration. The frequency distributions of closed times contained several exponential functions, suggesting multiple closed states. The time constants of the two shortest closed components were concentration independent. The time constant of the third-longest closed component in the short closed distribution decreased with increased concentration. In the long closed-time distribution, all three time constants decreased with increased concentration. Although the third-longest time constant in the short closed-time distribution was similar to the shortest time constant in the long closed-time distribution, it is not possible with the present methods to conclude that they are identical. Thus, at least five closed time constants exist. It is difficult to assign the longer closed component time constants to specific closed states since it was not possible to record from patches which definitely contained a single GABA receptor channel. However, since care was taken to include recordings from patches which had minimal (less than 1–3 %) occurrence of multiple openings, it is likely that multiple long closed states exist. Again, the constancy of the two shortest time constants over concentration suggests the presence of two closed states (C_1 and C_2) which probably occurred after GABA binding. The longer time constants decreased with concentration, suggesting that they occurred prior to the doubly liganded state. However, to further speculate on the location of GABA binding relative to closed states, more extensive analysis of burst and intraburst kinetics is required and is beyond the scope of the present paper.

Burst properties

GABA evoked bursts of one or more openings. ACh_n currents also occurred in bursts and bursts may be a fundamental behaviour of receptor-gated ion channels (Colquhoun & Sakmann, 1985). Burst frequency and mean burst duration increased with GABA concentration. However, the mean intraburst open time and the mean intraburst closed time did not vary with concentration. These properties suggest that bursts were produced by receptors which had bound GABA and that opening and closing within bursts occurred independently of GABA binding and unbinding.

The frequency distributions of burst durations contained three exponential components whose time constants were concentration independent. This suggests that the channel may open and close into bursts with at least three mean durations. An increase in mean burst duration with increased GABA concentration was achieved by a decrease in the relative proportion of bursts with short duration and an increase in the relative proportion of bursts with longer duration. The O_1 state and the short-duration bursts both had short time constants (mean 1·0 ms for both), and as concentration increased, the relative frequency of the O_1 state and short bursts decreased. Thus, it is likely that the short bursts represent single openings primarily to the O_1 state. The time constants for the two longer bursts were longer than the time constants for the O_2 and O_3 states. However, the relative frequency of occurrence of O_2 and O_3 open states and the two longer burst components increased with increasing GABA concentration. Thus, it is likely that the two longer burst

components were complex and were composed of groups of two or more openings primarily to the O_2 and O_3 states.

Comparison to other studies

Fluctuation noise analysis of GABA receptor currents from spinal cord neurones in culture revealed single- and occasionally double-Lorentzian characteristics of GABA open duration. The studies were limited by frequency response (usually 100–150 Hz) and thus could not resolve frequent brief openings or closings. None the less, channel opening duration was estimated to be 20 ms (McBurney & Barker, 1978), 30·7 ms (Mathers & Barker, 1980), 30·4 ms (Mathers & Barker, 1981), and 26·7 ms (Barker, McBurney & MacDonald, 1982).

Jackson *et al.* (1982) examined single-channel currents evoked by GABA (0·5 and 1·0 μM) with a megaohm on-cell patch recording system with an upper frequency limit of approximately 100 Hz. In three data sets, they found mean open durations of 10·0 and 21·5 ms for two one-exponential fits and 5·4 and 35·1 ms for a two-exponential fit. Sakmann *et al.* (1983) reported fitting an open-time distribution to two exponential functions with time constants of 2·0 and 29 ms (GABA, 2 μM). Mathers (1985) reported two-exponential fits for open times with time constants of 5·9 and 29·4 ms (GABA, 5 μM). For those two components, 46% of the area was in the first component. For 10 μm-GABA mean time constants of 4 and 24 ms were obtained, with the area not reported. Martin (1985) studied GABA channels recorded from *Ascaris suum* body muscle and reported open time constants of 2·1 and 46 ms for 3 μM-GABA and 4 and 101 ms for 10 μM-GABA. The shorter component area declined from 36 to 9% as GABA concentration was increased.

The kinetic properties of fast chloride channels from rat skeletal muscle have been well characterized (Blatz & Magleby, 1986). While not gated by GABA, they share many properties of GABA receptor channels. At least two open states with time constants of 0·5 and 1·5 ms (7·6 °C) were inferred from the open-time frequency distribution.

We have described three open-time exponential functions with time constants that were stable over a concentration range of 0·5–5 μM-GABA. The shortest open time constant was lower than previously reported in single-channel studies whereas the two longer time constants were between those reported in the studies above. Our ability to resolve three open time constants compared to one or two in previous studies may be due to differences in recording techniques or numbers of openings analysed. The increase in mean open duration with concentration was due to a shift in the relative proportion of openings from a shorter-lived state to longer stable states. There was no concentration-dependent effect on channel closing rates. This was in contrast to the report of Martin (1985) where the two open time constants resolved in *Ascaris suum* body muscle increased with GABA concentration. The difference between these findings may be due to the number of exponentials used in the fitting of the open-time distributions.

With single-channel recordings, bursts of openings may be resolved only as separate longer openings in systems with lower frequency resolution. Our longest burst-duration time constant of about 30 ms compares well to the 20–30 ms mean open times found in Lorentzian fits of noise analysis. For the present 2 μM-GABA

data, burst durations were best fitted to three exponential functions. The burst components were probably resolved as single open-time exponentials of 2·0–5·9 ms described in the studies above. The longest open time previously reported (17–29·4 ms) may represent our longest burst duration.

The mean closed time within bursts was independent of concentration and was similar to the properties reported for the ACh_n receptor (Colquhoun & Sakmann, 1985). We described two closed time constants (means, 0·24 and 2·0 ms) that were concentration independent. Martin (1985) described fitting closed-time distributions using three to four exponentials. At 3 μM-GABA, the time constants were 1·6, 40 and 1320 ms, and at 20 μM-GABA, the time constants were 5·6, 34, 310 and 1950 ms. Their results showed a decrease in the longer closed time constants with the increased GABA concentration, similar to our results. The occurrence of the additional time constant at 20 μM was felt to be due to desensitization. In contrast to the reported increase in the shortest time constant, we found no concentration dependence of the two shortest time constants.

Initial model

The durations of the observed channel open and closed times may be interpreted as sojourns in various states of a kinetic model represented by an unbound receptor, liganded receptor without channel opening and liganded receptor with channel opening (Colquhoun & Hawkes, 1982). We propose the following preliminary scheme for the GABA-receptor main conductance state that is composed of three open states, four closed states and two agonist binding sites, where A represents an agonist molecule, R a receptor, R′ an additional conformation of the doubly liganded receptor and * an open state.

$$\begin{array}{lll} 2A+R \rightleftharpoons A+AR \rightleftharpoons A_2R \rightleftharpoons A_2R' & & \text{Closed states} \\ \quad\uparrow\downarrow \qquad\quad \uparrow\downarrow \qquad\quad \uparrow\downarrow & & \\ A+AR^* \rightleftharpoons A_2R^* \rightleftharpoons A_2R'^* & & \text{Open states} \end{array}$$

The open states, O_1, O_2 and O_3, correspond to the bound open conformations AR*, A_2R^* and $A_2R'^*$, respectively. In this model, a singly liganded receptor may open to the brief open state (AR*).

The above scheme is consistent with the observation that as GABA concentration increased, the proportion of state AR* decreased relative to states A_2R^* and $A_2R'^*$. A burst may occur between an open state and its associated closed state. However, bursts of openings involving the doubly liganded states A_2R^* and $A_2R'^*$ would contribute the majority of current. This was suggested by the large increase in relative proportion of long bursts as concentration was increased. This must be considered a working model which is likely to be incomplete. It has been shown that models containing multiple open and closed states may be equally well described by a number of indistinguishable kinetic schemes (Blatz & Magleby, 1986). Thus, it will be difficult to determine a unique kinetic scheme for the GABA receptor channel.

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