

## KINETICS OF OPEN CHANNEL BLOCK BY PENICILLIN OF SINGLE GABA<sub>A</sub> RECEPTOR CHANNELS FROM MOUSE SPINAL CORD NEURONES IN CULTURE

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### SUMMARY

1. Reduction by penicillin of single  $\gamma$ -aminobutyric acid<sub>A</sub> (GABA<sub>A</sub>) receptor currents from somata of mouse spinal cord neurones in culture was investigated using the excised outside-out patch-clamp recording technique.

2. GABA (2  $\mu$ M) alone or with penicillin (100–5000  $\mu$ M) applied by pressure ejection from micropipettes evoked inward currents when patches were voltage-clamped at  $-75$  mV in symmetrical chloride solutions. Averaged GABA receptor currents were decreased in the presence of penicillin.

3. GABA receptor currents were recorded with at least two conductance states, a more frequent or main-conductance state of about 27 pS and a less frequent sub-conductance state of about 19–20 pS. The conductances of the two states were unchanged in the presence of penicillin. The kinetic properties of the main-conductance state were analysed and are summarized below.

4. Penicillin produced a concentration-dependent reduction of GABA receptor open properties by reduction of average GABA receptor channel open duration and an increase in channel opening frequency.

5. Penicillin shifted frequency histograms of GABA receptor channel open durations to shorter durations in a concentration-dependent manner. Three exponential functions were required to fit best the frequency histograms of open durations, suggesting that the channel had at least three open states. Penicillin produced a concentration-dependent reduction in the time constants obtained from the open duration frequency histograms.

6. Frequency histograms of GABA receptor channel closed durations could be fitted with five to seven exponential functions, suggesting that the channel had multiple closed states. In the presence of increased concentration of penicillin, there was a reduction in the relative frequency of brief gaps and the appearance of new closed time constants.

7. With increased penicillin concentration, GABA receptor channel burst frequency was unchanged, burst durations were increased, the number of openings per burst was increased and the per cent time open within a burst was decreased.

8. The results suggested that penicillin produced simple open channel blockade of

the GABA receptor channel. However, the experimental results also suggested that the association with and, perhaps, the dissociation of the blocker from its binding site were dependent upon the kinetic state of the open channel. Penicillin had faster association and slower dissociation rates when the channel was in an unstable, brief open kinetic state than when the channel was in a more stable, longer open kinetic state. Possible models for penicillin reduction of single GABA receptor currents were simulated by computer and analysed. Results obtained from simulated data were compared to those obtained from experimental data and a model that is consistent with the experimental observations is presented.

#### INTRODUCTION

Blockade of the pores of voltage-gated and ligand-gated ion channels has been interpreted to be an important mechanism for regulation of ion flow across neuronal membranes (Adams, 1976; Colquhoun & Hawkes, 1977; Hille, 1984). A channel block mechanism may be important in the action of drugs such as local anaesthetic block of sodium channels (Katz & Miledi, 1975; Adams, 1977) or of naturally occurring substances such as the proposed magnesium block of *N*-methyl-D-aspartate (NMDA) receptor channels (Nowak, Bregestovski, Ascher, Herbet & Prochiantz, 1984; Ascher & Nowak, 1988). However, the mechanism of ion channel blockade may not be simple and differentiation between allosteric regulation and channel blockade can be difficult (Mathie, Cull-Candy & Colquhoun, 1988). The microscopic kinetics of simple open channel block, non-simple or complex channel block and allosteric regulation are amenable to study using single channel recording and analysis techniques and can be used to infer mechanisms of channel regulation (Colquhoun & Hawkes, 1977, 1981, 1982).

For ligand-gated receptors, ion channel block has been only clearly demonstrated for the cation channel of the nicotinic acetylcholine (nACh) receptor. For example, simple open and closed channel block of nACh receptor currents has been shown to be produced by compounds such as QX222 (Neher & Steinbach, 1978; Neher, 1983), procaine (Katz & Miledi, 1975; Adams, 1977) and clonidine (Cull-Candy, Mathie & Powis, 1988). The anion selective  $\gamma$ -aminobutyric acid<sub>A</sub> (GABA) receptor shares similar activation or gating properties with the nACh receptor. Both receptors also appear to be derived from the same receptor family gene since subunits comprising the receptors share significant amino acid sequence similarities (Schofield, Darlison, Fujita, Burt, Stephenson, Rodriguez, Rhee, Ramachandran, Reale, Glencorse, Seeburg & Barnard, 1987; Barnard, Darlison & Seeburg, 1987). Thus, regulation of the two receptors also may be similar, although open channel block of GABA receptors has not yet been demonstrated using single channel recording and analysis techniques.

Penicillin has been shown to reduce GABA-evoked chloride current (Curtis, Game, Johnston, McCullough & MacLachlan, 1972; Davidoff, 1972*a, b*; Meyer & Prince, 1973; Macdonald & Barker, 1977, 1978; Chow & Mathers, 1986), has been shown to produce epileptogenic activity *in vitro* (Prince, 1968; Hochner, Spira & Werman, 1976; Dunn & Somjen, 1977; Krnjevic, Puil & Werman, 1977) and at sufficiently high concentrations, has provoked myoclonus and seizures *in vivo* (Ajimone-Marsen, 1969; Raichle, Kult, Louis & McDowell, 1971; Kao & Crill, 1972). Penicillin appears

to reduce GABA receptor currents in a non-competitive manner (Pickles & Simmonds, 1980), but the mechanism by which penicillin reduces GABA-evoked current is unknown. Allosteric regulation of the receptor, ion channel blockade or both have been proposed as mechanisms (Hochner *et al.* 1976; Macdonald & Barker, 1978; McBurney & Barker, 1978; Pickles & Simmonds, 1980). We have applied the single channel recording technique to investigate the mechanism by which penicillin regulates GABA receptor currents recorded from excised outside-out patches of mouse spinal cord neurones in culture. We demonstrate that the kinetics of penicillin reduction of single channel GABA receptor channel currents are consistent with simple open channel block of the receptor channel.

## METHODS

### *Cell culture*

To obtain spinal cord neurone cultures, timed pregnant mice were anaesthetized using CO<sub>2</sub> 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 2 ml of extracellular solution which consisted of the following, in mM: 142 NaCl, 8.1 CsCl, 1 CaCl<sub>2</sub>, 6 MgCl<sub>2</sub>, 10 glucose, 10 HEPES, (pH ~ 7.4). A high concentration of Mg<sup>2+</sup> in the extracellular solution was used to stabilize excised patches. The solution used in the micropipettes contained, in mM: 153 CsCl, 1 MgCl<sub>2</sub>, 10 Cs-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. All recordings were performed at room temperature (20–23 °C).

### *GABA and penicillin application*

A 1 mM-GABA (Sigma) stock solution in distilled water was diluted with extracellular solution to a final concentration of 2  $\mu$ M on the day of each experiment. Na-penicillin (benzyl-penicillin, Sigma) was dissolved and serially diluted in external bathing solution to concentrations of 100, 250, 500, 1000, 2500 and 5000  $\mu$ M at pH 7.4 on the day of the experiment. A mixture of GABA or GABA and penicillin was applied to the patch for at least 50 s via pressure ejection micropipettes that were moved to within 10  $\mu$ m of patches only during the time of each application.

### *Current recording*

Recording and pressure ejection micropipettes were constructed as previously described (Macdonald *et al.* 1989a). 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 8-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*

For the present study, data were accepted for analysis if only rare multiple openings (no evidence of more than three simultaneous openings) were detected during the application of GABA. If possible, all concentrations of GABA and penicillin were applied to each patch prior to patch disruption. The currents recorded had GABA receptor pharmacology. Using similar techniques, single GABA receptor currents obtained from excised outside-out patches of mouse spinal cord neurones in culture have been shown to be concentration-dependent (Macdonald *et al.* 1989a), reduced by bicuculline and picrotoxin (Macdonald *et al.* 1989a; Twyman, Rogers & Macdonald, 1989a) and enhanced by pentobarbitone (Macdonald, Rogers & Twyman, 1989b; Twyman *et al.* 1989a) and diazepam (Twyman, Rogers & Macdonald, 1989b).

Single channel recording and analysis techniques were as previously described (Macdonald *et al.*

1989a; Twyman, Rogers & Macdonald, 1990; Twyman & Macdonald, 1991). Single channel data were recorded on a VCR system and later digitized at 20 kHz with an 8-pole, 2 kHz Bessel filter interposed. System dead time and rise time were 70 and 130  $\mu$ 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 electrode 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, measured at the system output, was the time required for a measured square pulse to increase from 10 to 90% of its true amplitude.

Using locally written programs (R.E.T.), open and burst durations were placed into frequency histograms using linear binning, and closed durations were placed into frequency histograms using logarithmic binning. Linear frequency histograms were binned to minimize bin promotion errors according to methods previously described (McManus, Blatz & Magleby, 1987; Macdonald *et al.* 1989a). For curve fitting, open durations were binned into 0.1 ms bins with a range of 0.4–30 ms. Binning of open durations longer than twice the system rise time would provide accurate estimation of open durations that reached full amplitude. Logarithmic binning used a logarithmic time axis and a square-root ordinate transformation (Sigworth & Sine, 1987). Closed durations were binned using 10 bins/decade resolution with a 200  $\mu$ s lower limit. This lower limit would provide accurate estimation of closed durations that crossed below 50% of the full channel amplitude and were longer than twice the system dead time. Exponential curve fitting to determine the maximum likelihood estimates of time constants and areas (Colquhoun & Sigworth, 1983) was performed using locally written programs (Stat Library, IMSL, Inc., Houston, TX). 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 chi-square 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 using the likelihood ratio test (Horn & Lange, 1983; McManus & Magleby, 1989). Distributions of open, closed or burst durations were fitted over the same histogram ranges for GABA and each concentration of penicillin.

The open and closed durations reported in this study are observed durations. Due to the presence of unresolved or missed events and infrequent long duration events, the observed event durations can be greater than the true durations (Colquhoun & Sigworth, 1983). However, a simple correction of average open duration for missed short openings or unrecorded long 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 of each exponential component in the open duration histogram multiplied by the time constant of the component (corrected average open duration =  $a_1\tau_1 + a_2\tau_2 + a_3\tau_3$ ).

Total average current was defined as the average current per drug application and includes contributions from multiple simultaneous openings and openings to all conductance states. Average current was defined as the average current per drug application due to main-conductance state openings only.

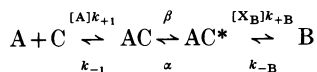
Since the data contained a small number of multiple simultaneous main-conductance state openings and openings to multiple conductances, it was difficult to determine unambiguously which closed durations represented the 'main-conductance state closed durations' or 'sub-conductance state closed durations'. For the purposes of this study, closed durations were analysed assuming that closed durations between main-conductance state openings were gated independently of other conductance states. With this analysis, non-main-conductance state openings were counted as closures while periods containing multiple openings were rejected (deleted). In addition, all closed durations between all openings (multiple-, main- and sub-conductance states) were analysed.

#### *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 analysis, a critical closed time,  $t_c$ , must be chosen such that all openings separated by closures less than  $t_c$  belonged within a burst, and bursts were separated by closures greater than  $t_c$ . Selection of a critical closed time in the presence of penicillin is provided in the Results section. Background for the analytic determination of bursts is as follows.

The analytical determination of bursts is crucial in separating kinetic modes of action. For

example in Scheme 1, a blocking compound at a concentration  $[X_B]$  can produce simple open channel block (Colquhoun & Hawkes, 1982; Colquhoun & Sigworth, 1983).



In this scheme which describes kinetic states of a single conductance level or state, A represents the agonist, C the closed unbound receptor, AC the closed bound receptor, AC\* the open bound receptor and B the open but blocked, non-conducting state. For the present purpose, it will be assumed that the time spent in state C is long, i.e. the association rate constant  $k_{+1}$  is small, and/or the agonist concentration  $[X_A]$  is low. Association rates for ligand-gated receptors may actually be quite high. The dwell time in state C will be considered to be long and may even incorporate a desensitized state. Since the states are known exactly in this example, bursts of openings may be arbitrarily defined as time spent in states to the right of AC. Thus, in the absence of a blocker, all bursts would be single openings to AC\* and produce a mean burst duration of  $\alpha^{-1}$  which is the mean dwell time in the open state. When blocker is added at a concentration of  $[X_B]$ , bursts will be composed of a series of openings (mean open duration of  $(\alpha + [X_B]k_{+B})^{-1}$  separated by closures (mean duration of  $k_{-B}^{-1}$ ) with a mean burst duration of  $(1 + ([X_B]k_{+B})k_{-B}^{-1})\alpha^{-1}$ . Mean open duration will thus decrease and mean burst duration will increase with increasing blocker concentration. If the dwell time in the blocked state is brief compared to time spent in C or AC (unblocking rate  $k_{-B}$  is large compared to the exit rates of states C or AC), the analytic separation of bursts could be performed by selecting a critical closed time  $t_c$  such that the expected proportion or number of errors (misclassification of closings in state C as being within bursts and misclassifications of blocks in state B as being outside of bursts) are equal and small. However, in the situation where the dwell time in states C or AC approaches or is less than the dwell time in the blocked state B, the analytical separation of bursts is difficult (Cull-Candy *et al.* 1988) and can produce a high rate of error. Analytically, the measured average burst duration may actually appear to decrease.

Now, consider the case if bursts were defined as oscillations of the open state with closed states to the right of C, but bursts began and ended with openings ('apparent bursts'). In the absence of blocker, the mean open time remains  $\alpha^{-1}$  and the mean burst duration becomes  $\alpha^{-1} + \beta[\alpha^{-1} + (\beta + k_{-1})](k_{-1})^{-1}$ . As above, addition of blocker decreases the mean open time to  $(\alpha + [X_B]k_{+B})^{-1}$ , and bursts are now composed of openings interrupted by a combination of state AC and blocked state B closings. If the dwell times in AC and B are much less than in state C, correct identification of bursts would not be difficult. Thus, some knowledge of microscopic gating kinetics should be determined before the effects of a blocker are characterized.

Single channel analyses of the GABA receptor currents have shown that the main-conductance state was gated to at least three open and ten closed states and a microscopic reaction scheme consistent with this has been proposed (Twyman *et al.* 1990). Since there are multiple states, the behaviour of a putative channel blocker may be complex. Thus, to analyse the effects of penicillin, we have attempted to model the effects of a blocker on the single channel properties of the GABA main-conductance state using Q-matrix methods and analysis of simulations of channel activity.

#### Q-matrix methods and simulated channel activity

Assuming Markovian behaviour, the Q-matrix method was used initially to determine rate constants for potential kinetic schemes (Colquhoun & Hawkes, 1981, 1982). Computer simulations of channel activity using these schemes were then used to compare kinetic schemes. Analysis of simulated channel activity was useful since the standard Q-matrix methods do not account for limited system resolution, and the probability density distributions predicted would not contain the effects of missed openings and closings. In addition, since transitions between adjacent closed states cannot be observed, simulation provided insights into the behaviour of closed duration distributions in a kinetic model where a potential channel blocker may also block directly from a closed state without transiting through an open state.

Software for simulations was written locally following methods similar to those previously described (Blatz & Magleby, 1986). The starting state was the closed, unbound state. The dwell time in a state was determined by generating an exponentially distributed random number (IMSL) whose mean was the reciprocal of the sum of the exit rates from that state. Relative exit rates gave the likelihood of entry to a connecting state and a second uniformly distributed random number was generated to determine which state was entered. To approximate data obtained experimentally, resolution was limited to openings longer than 250  $\mu$ s for true main-conductance

state openings (27 pS) using a 50% threshold crossing criterion in a system with a rise time of about 130  $\mu$ s and a sampling frequency of 20 kHz. Closed durations from 27 pS openings were limited to 150  $\mu$ s (three sample points) when considering the system dead time of about 70  $\mu$ s and requiring accurate resolution of closed duration (events greater than twice the system dead time). Models were simulated for single channel patches only. The contribution of the sub-conductance state was not analysed, and thus, the activity of the main-conductance state was considered to be independent of the sub-conductance state. Models were simulated for  $10^5$  observed open events for control GABA and each concentration of penicillin. Following generation of a series of openings and closings, the simulated data were analysed for open, closed and burst properties and open and closed durations curve fitted using the same methods applied to the experimental data at each concentration of penicillin. The results from analysis of simulated data were compared to the results of analysis of experimental data. Statistical methods of model discrimination using only open and closed duration distributions were not used (Akaike, 1974; Horn, 1987). In some situations they may be insufficient to discriminate among models (Korn & Horn, 1988; Colquhoun and Sigworth, 1983) or may lead to misleading conclusions when only a limited number of parameters are used. For this study, the following parameters were compared: average open, closed and burst durations, opening frequency, average number of openings per burst, average per cent time open within a burst, average closed time within a burst, and time constants and relative areas of open and closed distributions.

## RESULTS

### *Conductance properties*

Following hyperpolarization of outside-out patches to  $-75$  mV, GABA (2  $\mu$ M) evoked bursting currents in over 80% of patches (Fig. 1A). Only patches with reproducible activity and rare multiple simultaneous single channel currents were used for kinetic analysis. It was not possible to ascertain the actual number of channels per patch, but multiple simultaneous channel openings comprised only 2.8% of all detected openings.

Channel openings evoked by GABA occurred in bursts and with at least two current amplitudes suggesting at least two chord conductances (Fig. 1A) which were similar to conductances previously reported (Macdonald *et al.* 1989a). The larger conductance level or state (Fig. 1A, double asterisks) was recorded more frequently than the smaller conductance state (Fig. 1A, single asterisk). For all patches ( $n = 65$ ), current amplitudes evoked by GABA (2  $\mu$ M) were  $2.04 \pm 0.26$  pA and  $1.48 \pm 0.16$  pA at  $-75$  mV. Single channel currents reversed at about 0 mV. Channel chord conductances from control GABA recordings from all patches were  $27.2 \pm 3.4$  pS and  $19.7 \pm 2.2$  pS for the high and low conductance states, respectively. For detected openings from patches exposed to GABA alone, the larger or main-conductance state accounted for  $92.7 \pm 2.1$ % of the open duration and  $95.4 \pm 2.3$ % of the total current evoked.

GABA receptor channel current amplitudes at  $-75$  mV were unaltered by penicillin (100–5000  $\mu$ M) and averaged  $2.08 \pm 0.07$  pA and  $1.49 \pm 0.12$  pA ( $n = 65$  patches). This corresponded to chord conductances of  $27.7 \pm 0.09$  pS and  $19.8 \pm 1.6$  pS. In a representative patch, linear regression lines of a current–voltage relationship for channel evoked by GABA in the presence of penicillin (250  $\mu$ M) had slope conductances of 28.2 ( $r = 0.99$ ) and 19.2 ( $r = 0.98$ ) pS for the main-conductance (Fig. 2, inset, double asterisks) and the sub-conductance states (Fig. 2, inset, single asterisk), respectively.

The kinetic properties of the larger 27 pS or main-conductance state were determined since the main-conductance state provided  $88.5 \pm 3.1$ % of the openings

detected and  $95.0 \pm 2.6\%$  of the total current evoked. The kinetic properties of the sub-conductance state were not determined. A small shift in the proportion of openings to the two conductance states evoked by GABA in the presence of penicillin could not be excluded, although such a shift would have minimal effect on the total current evoked.

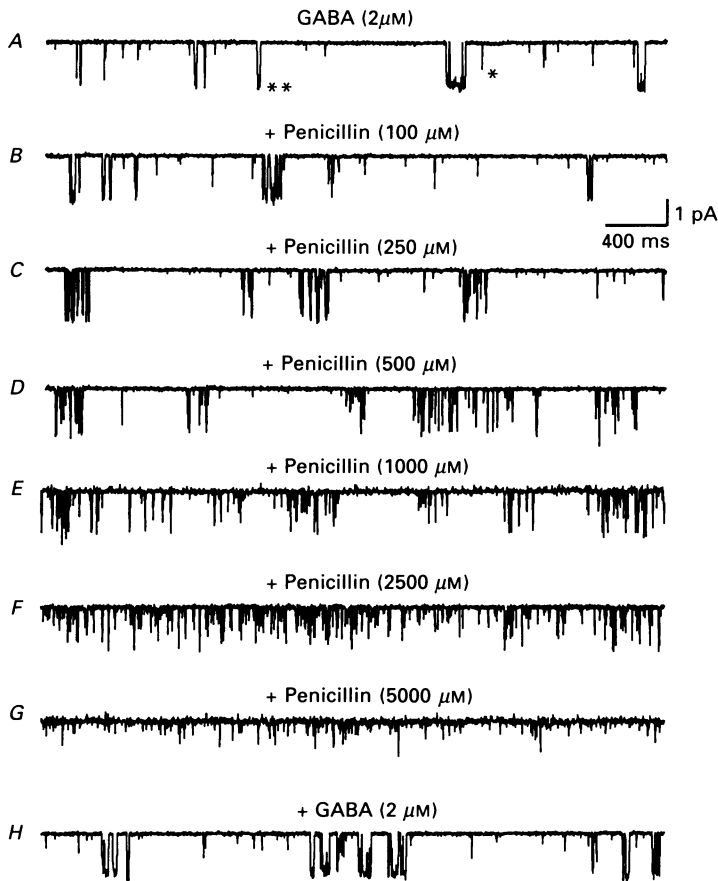


Fig. 1. Temporal characteristics of GABA-evoked single channel currents shown at low time resolution were altered in the presence of increased penicillin concentration. *A*, GABA ( $2 \mu\text{M}$ ) evoked bursting single channel inward (down going) currents with at least two current amplitudes when outside-out patches were voltage-clamped at  $-75 \text{ mV}$ . The larger  $2.04 \text{ pA}$  ( $27 \text{ pS}$ ) channel (double asterisk) occurred more frequently compared to a smaller  $1.48 \text{ pA}$  ( $19.7 \text{ pS}$ ) channel (single asterisk). *B–G*, penicillin ( $100\text{--}5000 \mu\text{M}$ ) altered temporal characteristics of GABA ( $2 \mu\text{M}$ )-evoked currents. The amplitude of brief openings was attenuated due to the graphics plotting routine. *H*, following wash-out ( $\sim 1 \text{ min}$ ), application of GABA ( $2 \mu\text{M}$ ) showed recovery of GABA-evoked currents. Tracings *A–E* and *H*, are data recorded from the same patch while *F* and *G* are representative traces from additional patches. Time and current calibrations apply throughout.

GABA alone evoked a total average current (all openings including multiple openings) of  $82 \pm 2.9 \text{ fA}$  per patch. Penicillin decreased GABA receptor currents in a concentration-dependent fashion (Table 1). At  $100 \mu\text{M}$ , penicillin reduced the total

average current by 33% to 55 fA, while at 5000  $\mu\text{M}$  the total average current was reduced by 80% to 16 fA.

### Open and closed properties

Penicillin (100–5000  $\mu\text{M}$ ) produced visually apparent alterations in the single channel currents evoked by GABA (Fig. 1). At low time resolution, openings in the

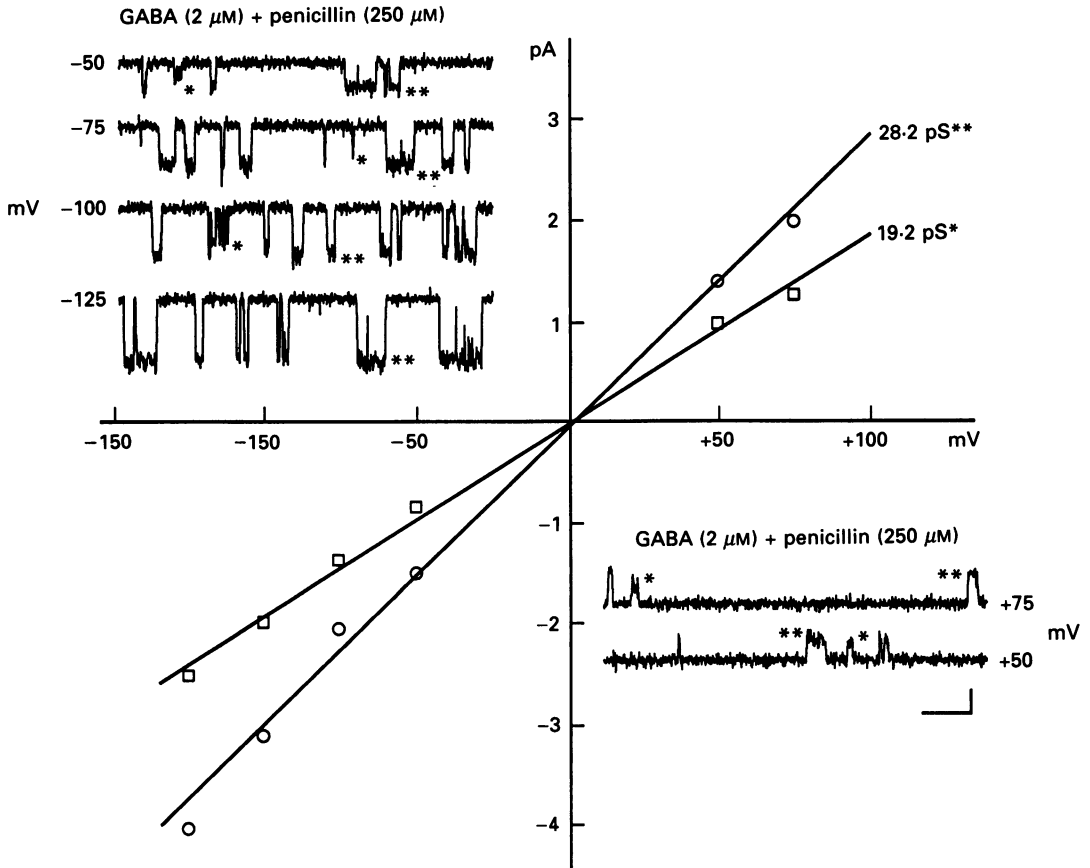


Fig. 2. Current-voltage relationship of single channel currents from a representative patch exposed to GABA (2  $\mu\text{M}$ ) plus penicillin (250  $\mu\text{M}$ ). Linear regression lines correspond to a slope conductances of 28.2 ( $r = 0.99$ ) and 19.2 ( $r = 0.98$ ) pS. Insets show examples of currents evoked by GABA (2  $\mu\text{M}$ ) plus penicillin (250  $\mu\text{M}$ ) at the membrane potentials indicated. The examples were selected to demonstrate current amplitude relationships and were not necessarily representative of kinetic activity. Time (25 ms) and current (1 pA) calibrations apply to both insets.

presence of penicillin clustered together (Fig. 1*B-G*) when compared to openings evoked by GABA alone (Fig. 1*A, H*). At increased time resolution when compared to GABA alone (Fig. 3*A, a-e*), penicillin reduced the duration of openings and increased the duration of closings between groups of openings (Fig. 3*B, a-e*).

GABA (2  $\mu\text{M}$ ) evoked channel openings with an average duration of 3.66 ms which was decreased to 3.28 when corrected for undetected openings (see Methods and





Table 1). Mean channel open duration was decreased by penicillin in a concentration-dependent manner from 3.52 ms at 100  $\mu\text{M}$  to 0.90 ms at 5000  $\mu\text{M}$ . Corrected average open durations decreased from 2.67 to 0.54 ms over the same concentration range (see Methods and Table 1). Corrected average open duration was decreased 19% by

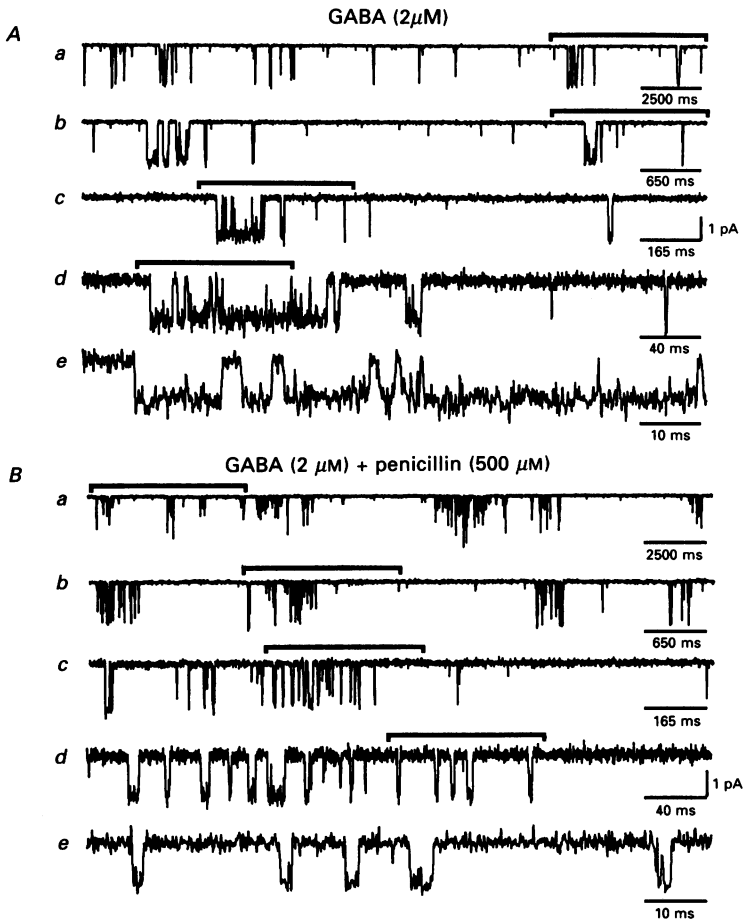


Fig. 3. Single channel currents are shown at increasing time resolution for GABA (2  $\mu\text{M}$ ) (A, a-e) and GABA (2  $\mu\text{M}$ ) plus penicillin (500  $\mu\text{M}$ ) (B, a-e). The portion outlined for each tracing is presented expanded in time in the tracing beneath it. Attenuated brief channel openings seen at lower temporal resolution may be seen at true amplitudes at higher temporal resolution. Time calibration for each trace is shown on the right below the trace. Current calibration applies throughout.

100  $\mu\text{M}$ -penicillin and 89% by 5000  $\mu\text{M}$ -penicillin. Thus, reduction of GABA receptor current by penicillin was primarily due to a decrease in open duration.

#### *Stationarity of GABA receptor currents*

Average open durations of the main-conductance state varied little when the data at each concentration were divided into six consecutive 10 s periods and analysed from 1 s after the beginning of GABA application (not illustrated). For GABA alone, the variance of the observed average open duration was 2.3% over the six periods.

In the presence of penicillin, the open duration variance averaged 2.5%. This demonstrated that the average closing rates of the main-conductance state were stationary in the presence or absence of penicillin. Opening frequency decreased from the beginning of GABA application. For GABA alone, opening frequency decreased with a slope of  $-0.17$  ( $r = 0.88$ ) over the sixty seconds from the beginning of application. In the presence of penicillin, the opening frequency decreased with an average slope of  $-0.19 \pm 0.8$  (averaged  $r = 0.66 \pm 0.13$ ). The basis for the decrease in opening frequency is unknown but was likely due to an increase in long closed durations, perhaps from desensitization.

#### *Frequency distributions of open durations.*

To determine the basis for the decrease in average open duration by penicillin, open durations were collated into frequency histograms. Open durations were shifted to shorter durations in the presence of penicillin (Fig. 4A). The open duration frequency histograms for GABA alone were best fitted over a histogram range of 0.4 to 50 ms with a sum of three exponential functions. The exponential components were designated 1, 2 and 3 for the shortest to the longest time constants, respectively (Fig. 4B). The time constants were 0.78, 3.1 and 8.6 ms for components 1 to 3 and were similar to those reported previously (Macdonald *et al.* 1989a; Twyman *et al.* 1990). The small differences are due to improved recording techniques, increased frequency bandwidth and increased sampling frequency used in the present study. At all concentrations of penicillin (100–5000  $\mu\text{M}$ ), the open duration frequency histograms were best fitted with sums of three exponential functions. The open dwell time constants decreased in the presence of increased penicillin concentration (Fig. 4B). At 100  $\mu\text{M}$ , they were 0.47, 2.9 and 5.4 ms for components 1 to 3. At 5000  $\mu\text{M}$ , the time constants were decreased to 0.28, 0.61, and 1.2 ms. The longer duration open time constants were decreased more than the shortest time constants as penicillin concentration was increased. Estimation of the relative proportions of the components was difficult, and tended to be correlated with the variability of the time constant estimates. The relative proportions of the components remained approximately the same in the presence or absence of penicillin (Fig. 4B). Penicillin primarily decreased average open duration by decreasing mean dwell time in each open component.

#### *Frequency distributions of closed durations*

Since the number of sub-conductance state and multiple openings were relatively few compared to the number of main-conductance state openings, the distributions of closed durations between all openings (main- and sub-conductance states) were not greatly different from the distribution of closed durations between main-conductance state openings (not illustrated). Sub-conductance state openings were infrequent and tended to occur in isolation. The distributions of closed durations between sub-conductance state openings contained relatively long closed durations and were few in number compared to those between main-conductance state openings. Results from the analysis of closed durations between main-conductance state openings are presented.

For GABA in the absence of penicillin, the profile of the closed distribution was smooth and suggested the presence of multiple exponential components (Fig. 5).

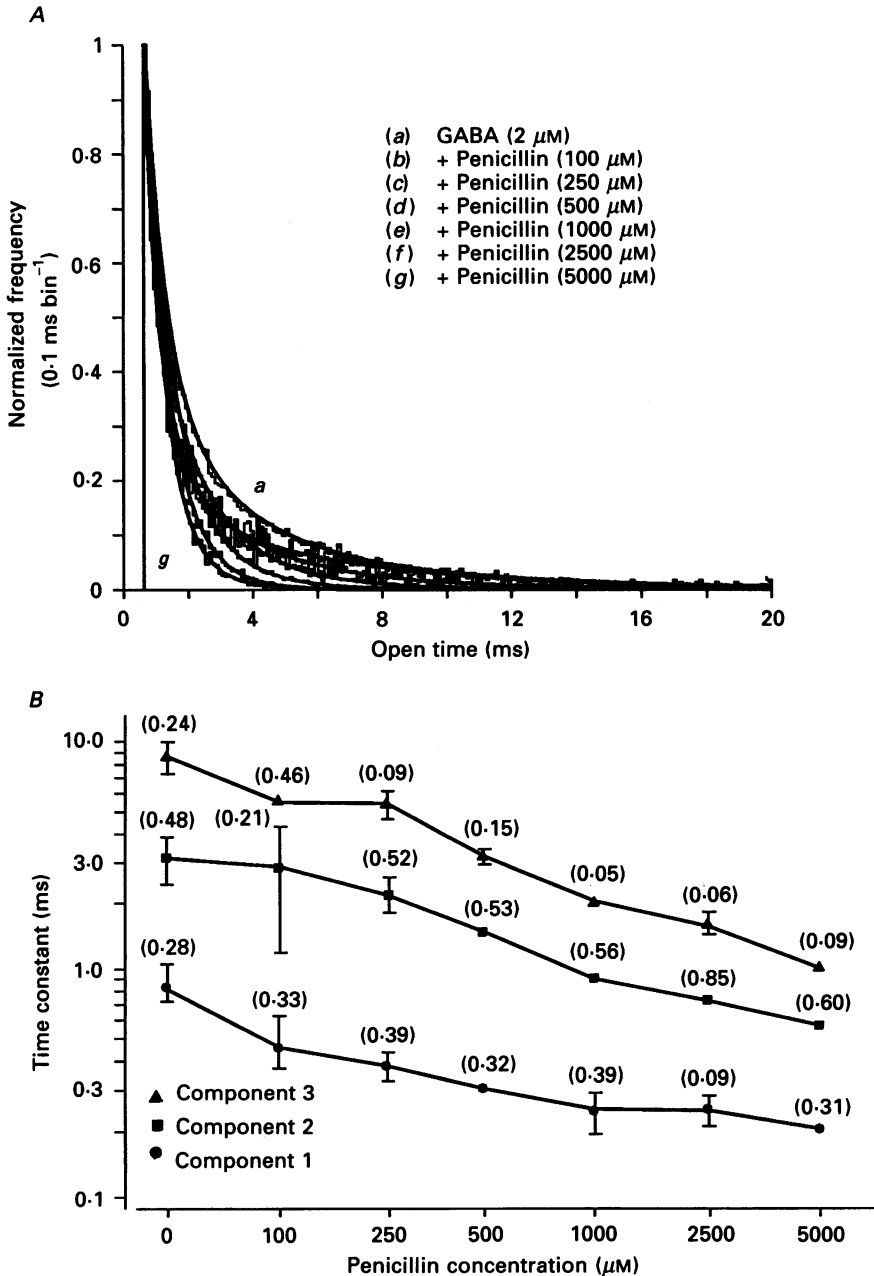


Fig. 4. *A*, normalized linear-binned open duration frequency distribution histograms for GABA (2  $\mu\text{M}$ ) and GABA (2  $\mu\text{M}$ ) plus penicillin (100–5000  $\mu\text{M}$ ). Openings were placed into 0.1 ms bins and displayed for clarity over a range of 0.4–20 ms. Distributions were normalized and overlaid to display relative frequency distributions. Histograms were best fitted with the normalized sum of three exponential distributions and curves were drawn according to the fits (see text). The distribution farthest to the right (*a*) corresponds to GABA (2  $\mu\text{M}$ ). GABA (2  $\mu\text{M}$ ) plus penicillin (100–5000  $\mu\text{M}$ ) progressively shifted open durations to shorter openings and shifted the distributions to the left (*b*–*g*). The distribution farthest to the left corresponds to GABA (2  $\mu\text{M}$ ) plus penicillin (5000  $\mu\text{M}$ ) (*g*).

Visual inspection of the closed duration distributions revealed that the closed distribution profiles were progressively modified by increased concentrations of penicillin. At the higher penicillin concentrations, the closed distributions were dominated by closings of medium duration. All closed duration distributions were best fitted with sums of five to six exponential functions (Fig. 6). The exponential components were designated 1 to 5 for the five shortest time constants. Since the exact number of channels active in each patch was unknown, the significance of 'long' closed durations and the longer closed time constants was unclear. The time constants for components 1 and 2, about 0.2 ms and 1.5 ms respectively, did not change in the presence of low concentrations of penicillin. At higher penicillin concentrations, the two components became more difficult to resolve. This was due to a progressive decrease in the relative proportions of these components as penicillin concentration was increased (Fig. 6). The middle components varied somewhat at low penicillin concentrations, but at higher penicillin concentrations two predominant components with time constants of about 6 and 20 ms were resolved. The proportions of these components were seen to increase as penicillin concentration was increased and accounted for about 85% of the total closed distribution area. Thus, penicillin appeared to decrease the likelihood of brief gaps evoked by GABA. This observation may be explained by at least two different mechanisms. Penicillin may allosterically reduce the entry (transition rate) into brief closed states or transitions to the brief closed states may be decreased in probability (such as exit from a closed state to an adjacent blocked closed state would only be recorded as a longer closed dwell time). These alternatives will be considered later.

#### *Analytical determination of bursts by a critical closed time*

Since short closed durations were considerably altered in the presence of penicillin, the analytical determination of bursts using a constant short critical closed time would likely produce substantial misclassifications. A critical closed time of 5 ms was previously described for the determination of the end of a burst evoked by low concentrations of GABA (0.5–5  $\mu\text{M}$ ) (Macdonald *et al.* 1989a). GABA-evoked bursts analysed in this fashion primarily contained oscillations of longer open states between brief closed states (about 0.2 and 2 ms lifetimes and 59% of the relative proportion in the closed duration distribution) (Twyman *et al.* 1990). In the presence of increasing penicillin concentration, the two shortest closed time constants diminished in relative proportion and were replaced by the longer time constants. Therefore a longer critical closed time was chosen for these analyses. Such a closed time may include large bursts of openings (including the singly liganded state) or clusters of openings. Inspection of closed durations from the closed duration histograms and of results from curve fitting of the closed duration histograms revealed that the majority of closings were generally shorter than 100 ms. Indeed, virtually all of the closed durations resulting in the 6 and the 20 ms time constants

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*B*, time constants and relative areas of open frequency distributions for GABA (2  $\mu\text{M}$ ) and GABA (2  $\mu\text{M}$ ) plus penicillin (100–5000  $\mu\text{M}$ ). Time constants for exponential components 1 to 3 correspond to functions with the shortest to the longest time constants, respectively (see key). Error bars represent likelihood intervals. For each of the time constants, relative area estimates without error ranges are shown in parentheses above each time constant point.

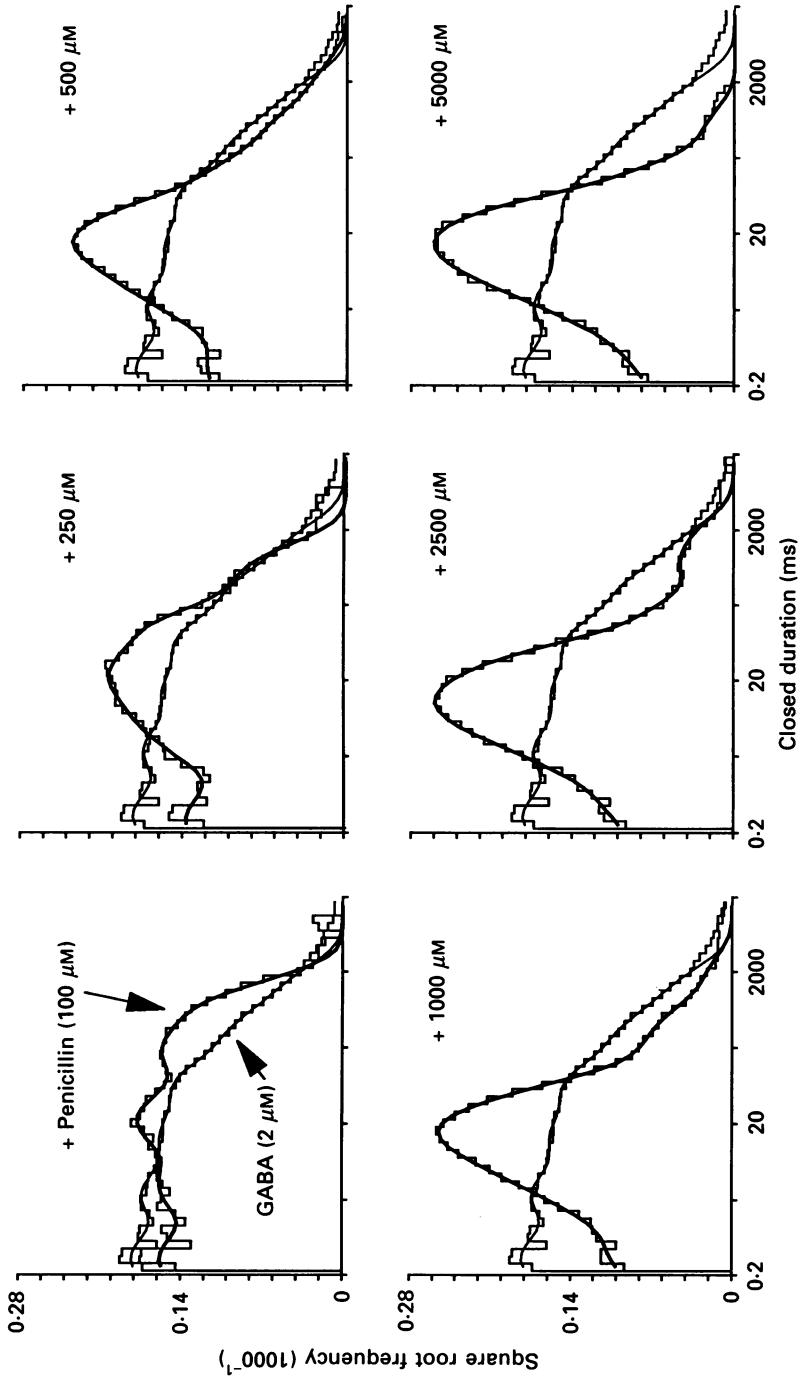


Fig. 5. Logarithmic-binned frequency histograms of closed durations of GABA ( $2 \mu\text{M}$ ) and GABA ( $2 \mu\text{M}$ ) plus penicillin ( $100\text{--}5000 \mu\text{M}$ ). The distribution for GABA ( $2 \mu\text{M}$ ) was overlaid at each penicillin concentration to display relative frequency distributions. Histograms were best fitted with the sum of five to six exponential functions and curves were drawn according to the fits (see text).

would be less than 100 ms. Thus, a critical closed time of 100 ms was selected to include large bursts or clusters of openings that would likely be due to openings and closings from the singly or doubly bound receptor.

*Burst properties*

GABA alone evoked bursts with average duration of 82 ms (Table 1). Mean durations of GABA-evoked bursts increased with increased concentrations of

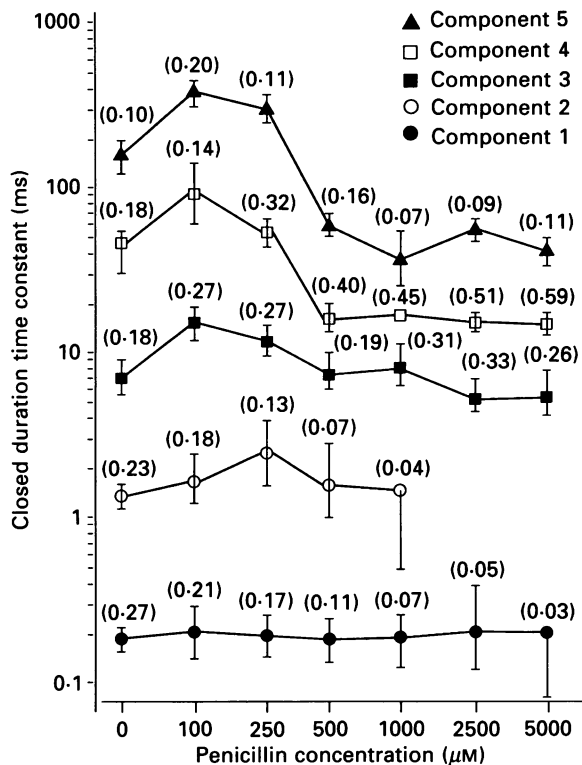


Fig. 6. Time constants and relative areas of closed duration frequency distributions for GABA (2 μM) and GABA (2 μM) plus penicillin (100–5000 μM). Time constants and relative areas for exponential components 1 to 5 correspond to the five shortest time constants (see key and text). Error bars for the time constants represent likelihood intervals ( $m = 2$ ). For each of the time constants, relative area estimates without error ranges are shown in parentheses above each time constant point.

penicillin from 58 ms at 100 μM to 220 ms at 2500 μM-penicillin. At 5000 μM-penicillin, the average burst duration was slightly decreased to 175 ms. Burst frequency was not changed by penicillin (100–5000 μM) and ranged from 1.4 to 2.1 per second. The per cent time open within a burst was 25.4% in control and decreased from 23.6% in 100 μM to 3.9% in 5000 μM-penicillin. The average number of openings detected within a burst evoked by GABA was 4.5 and increased with penicillin concentration from 3.9 at 100 μM to 14.0 openings per burst at 2500 μM-penicillin. Average number of openings per burst decreased to 7.8 at 5000 μM-

penicillin. The average duration of closed periods within a burst evoked by GABA was 14.1 ms and the durations were generally slightly longer in the presence of penicillin, ranging from 15.4 to 25.1 ms.

Thus, penicillin altered properties of bursts evoked by GABA in a concentration-dependent manner. The primary effects included a decrease in per cent time open within a burst and an increase in burst duration and openings per burst. At the highest penicillin concentration (5000  $\mu\text{M}$ ), the average burst duration and openings per burst were decreased compared to those obtained for 2500  $\mu\text{M}$ -penicillin.

#### *Voltage dependence of penicillin reduction of GABA receptor currents*

At positive patch potentials, penicillin decreased the average GABA receptor current to a greater degree than at negative patch potentials (not illustrated). However, due to difficulties in maintaining stable patches and obtaining high signal-to-noise ratio recordings at positive potentials, only limited analysis of these data could be performed. At low concentrations of penicillin (100–500  $\mu\text{M}$ ), average burst durations were increased compared to control, but at higher concentrations (1000–5000  $\mu\text{M}$ ), average burst durations were decreased compared to control. In some recordings at the highest penicillin concentration (5000  $\mu\text{M}$ ), no channel openings could be detected at +75 mV membrane potential while openings could be detected when the patch was hyperpolarized to -75 mV.

#### *Q-matrix computer simulation of potential kinetic schemes*

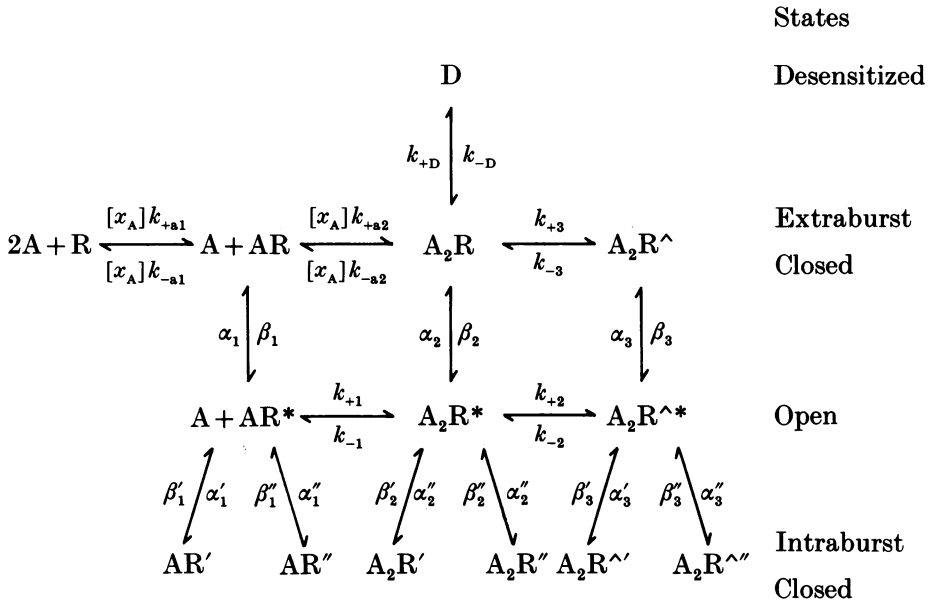
Simple linear and cyclical gating schemes for the GABA receptor have been shown to be inadequate (Macdonald *et al.* 1989a; Weiss & Magleby, 1989; Twyman *et al.* 1990). Using an extension of the Monod-Wyman-Changeux model, a microscopic kinetic gating scheme for the main-conductance state of mouse spinal cord neurone GABA receptors activated by low concentrations of GABA has been proposed (Twyman *et al.* 1990). This gating scheme without desensitization has been shown to be a reasonable working model for the GABA receptor gated by low concentrations of GABA at equilibrium (Macdonald *et al.* 1989a; Twyman *et al.* 1990). The model is certainly incomplete since studies examining saturating concentrations of GABA and phosphorylation of the receptor suggest a role for desensitization or inactivation (Akaike, Inomata & Tokutomi, 1987; Cash & Subbarao, 1987; Gyenes, Farrant & Farb, 1988; Chen, Stelzer, Kay & Wong, 1990; Porter, Twyman, Uhler & Macdonald, 1990). The properties of desensitization of single channels have yet to be characterized. Studies in this laboratory have suggested that long-lived desensitized states involved primarily fully liganded receptors (authors' unpublished observations). For the present study, a doubly bound long-lived desensitized state was incorporated into the model. It is acknowledged that characteristics of the desensitization are still undefined and may involve several forms. The presence of this desensitized state in this model would produce long-lived closed sojourns that would be interrupted by bursts or clusters of openings similar to those seen with the nACh receptor (Sakmann, Patlak & Neher, 1980). It would also account for the slow run-down or decrease in open frequency observed when GABA was applied.

A modified version containing a doubly bound desensitized state (D) is summarized below. In this sequential binding scheme (Scheme 1A), A represents agonist, R, R', R''

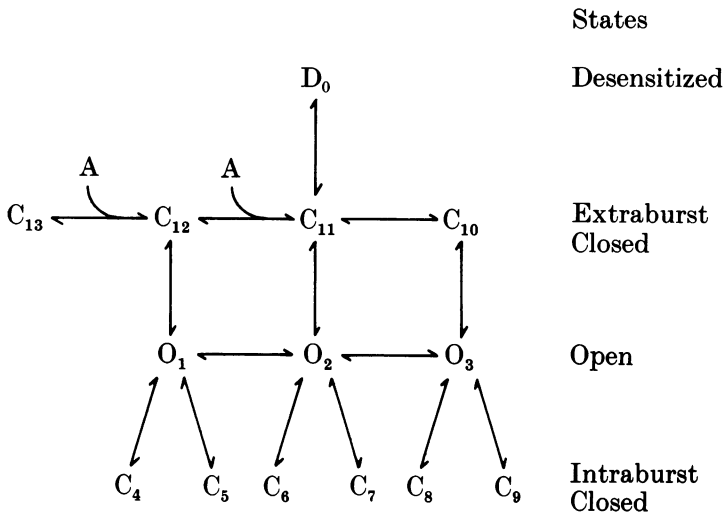


and  $R'$  represent receptor conformations and  $*$  represents an open state of the receptor. Transition rates are represented by  $k$ ,  $\alpha$  and  $\beta$  and agonist concentration is represented by  $[X_A]$ . Although it is recognized that equivalence of the binding sites and cooperativity of GABA binding have not been addressed, a non-cooperative binding model will be assumed for this study. The microscopic equilibrium constants are thus  $K_1 = 2k_{-1}/k_{+1}$  and  $K_2 = k_{-2}/2k_{+2}$ , and  $K_1 = K_2$ .

The open and closed states of Scheme 1A are numbered in Scheme 1B to simplify



Scheme 1A



Scheme 1B

discussion. For Scheme 1B, rate constants corresponding to microscopic transition rates between connected states  $i$  and  $j$  will be designated  $k_{ij}$  (see Appendix).

The open states correspond to the bound states 1, 2 and 3, respectively. Closed extraburst states correspond to the unbound state 13, singly bound state 12 and doubly bound states 10 and 11. Intraburst closed states correspond to singly bound states 4 and 5, and doubly bound states 6, 7, 8 and 9. In this model, the singly liganded receptor may open to the brief open state 1 and the doubly liganded receptor may open to the longer lived open states 2 and 3. Once in an open state, open states 1, 2 and 3 primarily oscillate with adjacent brief closed states 4 and 5, 6 and 7, and 8 and 9, respectively. States 4, 6 and 8 have the same or very similar exit rates and correspond to component 1 of the closed distribution. States 5, 7 and 9 have the same or very similar exit rates and correspond to component 2 of the closed distribution. The properties of these brief intraburst closed states have been described previously (Twyman *et al.* 1990). The bases for the brief closed states are uncertain but they may represent transient blocking of the channel or intrinsic closures of the channel (Macdonald *et al.* 1989a; Twyman *et al.* 1990). Oscillations primarily between these states and the adjacent open state may produce bursts of openings separated by long closures that include combinations of closed states 10 through 13. Combinations of bursts would produce clusters of openings between longer duration closures that primarily involved closed state 13.

In complex gating schemes, current could be decreased by several mechanisms: decreased opening frequency, decreased dwell time in the open states, and/or reduced probability of opening into longer more stable open states. From several alternative mechanisms, we initially chose to examine an open channel blocking mechanism for several reasons. First, penicillin produced a concentration-dependent reduction of the time constants of *each* of the three open states. As was observed with GABA-evoked openings in the presence of penicillin, there was a concentration-dependent decrease in the open time constants. A compound that modified GABA affinity or entry into the open state would not be expected to alter open state time constants. Second, burst properties were altered in a manner suggestive of open channel block. Burst duration and openings per burst increased as blocker concentration increased. Third, closed duration analysis revealed a penicillin-dependent decrease in the relative proportion of both of the briefest intraburst closed time constants. The two briefest intraburst time constants have been shown to be GABA concentration-invariant and appear to be distal to the open states. As open channel blocker would reduce the proportion of these two closed time constants (representing closed states 4 to 9) as blocker concentration increased since the exit rate from the open state to the blocked state would be increased relative to that of the brief closed states. Thus, the overall single channel behaviour evoked in the presence of penicillin had characteristics suggestive of a simple open channel block mechanism. However, the GABA receptor channel opened to at least three open states, and the kinetic mechanisms of blockade for each of these states were not clear. To further understand how penicillin reduced GABA receptor current, model simulation was begun by incorporating a simple open channel block of each of the open states (Scheme 2).



experimental data, Model 1 appeared to be incorrect based upon analysis of open and closed duration time constants. Open and closed durations of the simulated GABA-evoked activity of Model 1 were collated into frequency histograms and were analysed in fashion similar to the experimental data. Three time constants obtained from the open duration distributions decreased with penicillin concentration (not illustrated), but the relative proportions of components 2 and 3 were greater than those observed in the experimental data. Also, the concentration dependent decrease in the time constants was excessive for components 2 and 3 and insufficient for component 1 (not illustrated). Time constants obtained from the closed time distributions were dominated (over 90%) by the contribution from a 16 ms time constant which was not observed in the experimental data. This 16 ms time constant represented the observed contribution of the dwell times in the blocked states 14 through 16 since the unblocking rates from each of these states were identical.

The penicillin concentration-dependent decrease in the open state time constants found in the experimental data suggested that penicillin may not block each open state equally. Thus, the association rates  $k_{+B}$  might be different for each open state. This implied that the association of penicillin with its binding site was dependent on the kinetic state of the receptor. Physically, this might mean that the accessibility of penicillin to its binding site was dependent upon the open conformation of the channel.

The apparent closing rates for all three open states increased in a log-linear fashion (by a factor of approximately 0.75 per doubling of penicillin concentration). For open components 1, 2 and 3, blocker association rates were  $28.2 \times 10^5$ ,  $9.2 \times 10^5$  and  $3.7 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ , respectively. These rates implied that the penicillin binding site was most accessible and the potential for blockade of the channel was greatest when the channel was in the most unstable open conformational state. Rate constants for this model are provided in the Appendix as blocked Model 2. The computer simulation results for Model 2 were more similar to the experimental data than the results of Model 1. Frequency histograms of open durations and the time constants and relative areas obtained for the histograms (not illustrated) were more similar to the experimental data than Model 1. However, this model also appeared to be incorrect. At the highest penicillin concentrations, average burst durations and openings per burst continued to increase with concentration similar to Model 1 but contrary to the experimental result. Also, the closed time distributions continued to be dominated by a single time constant of about 16–17 ms and was due to identical dissociation rates of the blocked states.

It was noted that at the highest penicillin concentrations, two time constants (about 6 and 20 ms) dominated the closed duration distribution. If the dissociation of penicillin was independent of open state conformation, the unblocking (re-opening) rate should have been the same for each of the open states. Thus, as was found for Models 1 and 2, the closed duration distribution should have been dominated by one closed time constant that was associated with the unblocking rate. Conversely, if the unblocking rates were open state dependent and non-identical, three closed time constants should be found to increase in proportion as penicillin concentration was increased. The relative contribution of these three blocked states would be dependent on the relative number of open-blocked state transitions that



occurred for each open state. The Q-matrix results predicted that for Model 2, the relative frequency of openings from open states 2 and 3 would predominate. This was particularly true at the highest penicillin concentrations where most of the openings (over 98%) due to state 1 would be so brief as to be undetected. These unobserved openings from state 1 would produce long closed durations and would contribute to an apparent increase in baseline noise. Thus, the 6 and 20 ms closed duration time constants of the experimental data may have been related to blocked states associated with open states 2 and 3. Q-matrix results of Model 2 predicted that the relative proportion of openings due to state 2 would be about twice that due to state 3. The relative proportion of the component with the 20 ms closed time constant was about twice that of the 6 ms time constant. This suggested that blocked state 15 which associated with open state 2 resulted in the 20 ms time constant and blocked state 16 which was associated with open state 3 resulted in the 6 ms time constant. Although blocked states were isolated from other closed states in Scheme 2, the actual dwell times in each of the blocked states would not correlate exactly to the measured time constants. This was due to the limited frequency response of the recording system which caused brief openings and closings to remain undetected. Therefore, computer simulations were used to evaluate these effects, and the unblocking rates were adjusted until the properties of the model approximated the experimental data (Model 3 in Appendix).

The open, closed and burst property results of Model 3 were compared to the experimental data (Table 2 compare to Table 1) and approximated the experimental data more closely than Models 1 and 2. Rather than calculating a correction of average open duration, the average open duration of all opening predicted by the model are presented. Similarly, both observed and predicted opening frequencies are presented. The predicted opening frequencies increased with penicillin concentration, whereas the observed frequencies decreased at the higher penicillin concentrations in a fashion similar to that observed experimentally. Contrary to Models 1 and 2, burst durations and openings per burst plateaued at the highest penicillin concentrations, similar to that observed experimentally. The frequency distributions of open (Fig. 7A) and closed durations (Fig. 8) also were similar to those of experimental data. Open (Fig. 7B) and closed (Fig. 9) time constants and relative areas better approximated the experimental data than Models 1 and 2. The closed distributions of the highest penicillin concentrations were dominated by time constants of about 7 and 20 ms which represented the observed contribution of the dwell times in blocked states 16 and 15, respectively. Estimation of the contribution of the blocked state 14 was difficult since many of the openings from the brief open state 1 were unobserved at the highest penicillin concentrations. The model in Scheme 2 predicts that ten time constants should be present in the closed duration distributions, but only five or six components were resolved in both the experimental and simulated data. This was because the relative contribution of the other components to the total area of the density distribution was small (less than 1–5%).

Based upon curve fits at the lower penicillin concentrations and estimates of the number of missed openings due to the limited resolution of the system, a dissociation rate of about  $55 \text{ s}^{-1}$  was estimated for blocked state 14. With these results, three microscopic  $K_{\text{ps}}$  of 26, 109 and  $545 \mu\text{M}$  were estimated for the blocking of open states

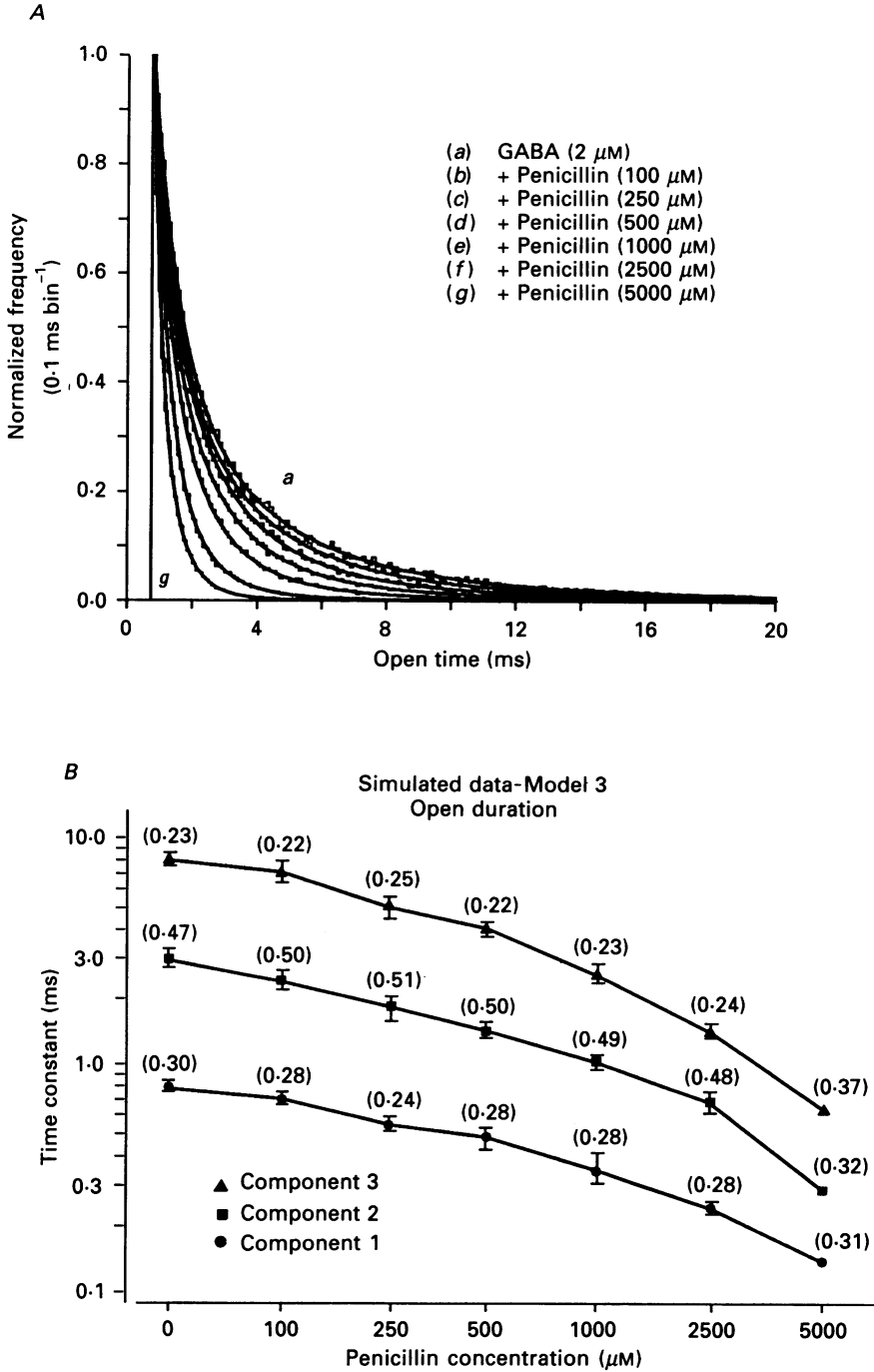


Fig. 7. Normalized linear-binned open duration frequency distribution histograms (A) and open distribution time constants and relative areas (B) of simulated channel activity for GABA (2 μM) and GABA (2 μM) plus penicillin (100–5000 μM) predicted by Scheme 2, Model 3 (see text). Data are presented in a fashion similar to Fig. 4.

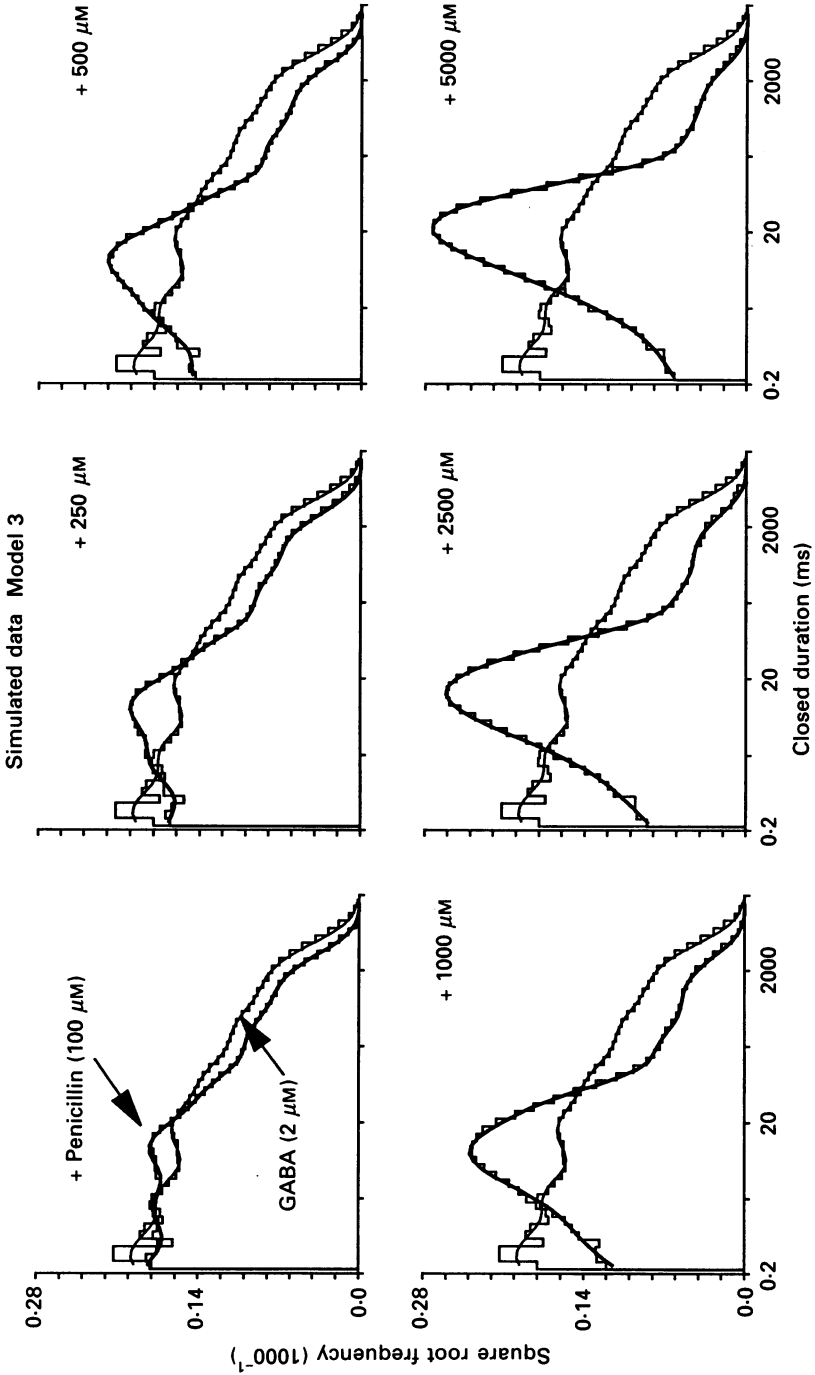


Fig. 8. Logarithmic-binned closed duration frequency distribution histograms of simulated channel activity for GABA (2  $\mu\text{M}$ ) and GABA (2  $\mu\text{M}$ ) plus penicillin (100–5000  $\mu\text{M}$ ) predicted by Scheme 2, Model 3 (see text). Data are presented in a fashion similar to Fig. 5.



1, 2 and 3, respectively. Although the  $K_B$  for the briefest open state 1 was in the low micromolar range, the relative effect on current reduction of this state was low. This was due to relatively low frequency of occurrence and brief duration and, thus, small amount of current that occurred from the brief open state. For  $2 \mu\text{M}$ -GABA in the unblocked model, the briefest open state was responsible for less than 6% of the total current from all open states.

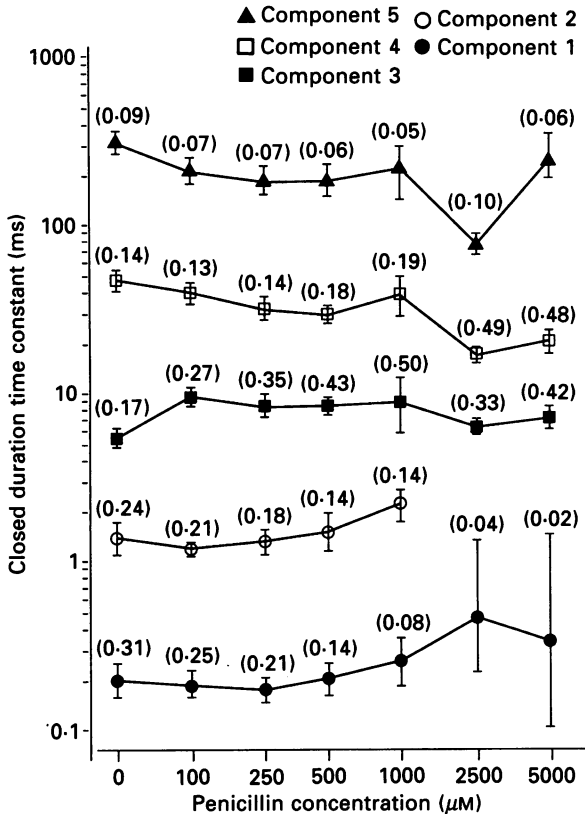


Fig. 9. Closed duration frequency distribution time constants and relative areas of simulated channel activity for GABA ( $2 \mu\text{M}$ ) and GABA ( $2 \mu\text{M}$ ) plus penicillin ( $100\text{--}5000 \mu\text{M}$ ) predicted by Scheme 2, Model 3 (see Text). Data are presented in a fashion similar to Fig. 6.

DISCUSSION

Conductance

For patches held at  $-75 \text{ mV}$  in symmetrical chloride solutions, two predominant current amplitudes were evoked by GABA with average chord conductances of about 27 and 19–20 pS. The dominant or main-conductance state was the 27 pS state and was similar to that previously reported (Bormann, Hamill & Sakmann, 1987; Smith, Zorec & McBurney, 1989; Macdonald *et al.* 1989*a*). The chord conductances of the main- and sub-conductance states were unchanged in the presence of penicillin

(100–5000  $\mu\text{M}$ ), and there was not a major shift in the relative proportion of the main- and sub-conductance states. Thus, mechanisms that reduce ion channel conductance, such as an apparent reduction of conductance due to rapid blocking and unblocking of the channel postulated for proton blockade of sodium channels (Woodhull, 1973), or a modification of charge configuration within the pore was unlikely (Sakmann, Methfessel, Mishina, Takahashi, Takai, Kurasaki, Fukuda & Numa, 1985; Mishina, Imoto, Noda, Takahashi, Numa, Methfessel & Sakmann, 1986). For agents that reduce current without an alteration of channel conductance, slower channel block or allosteric regulation of channel gating have been suggested as mechanisms for the nACh receptor channel (Adams, 1977; Neher & Steinbach, 1978; Cull-Candy *et al.* 1988). Similarly, the regulation of temporal kinetics of the main-conductance state was the major factor for GABA receptor current reduction by penicillin.

#### *Single channel open properties*

The open properties of single channel GABA receptor currents were penicillin concentration-dependent. The decreased average single channel current in the presence of penicillin was due to a decrease in time spent in the open state and was consistent with the decreased GABA-evoked current by penicillin observed in cultured central neurones (Macdonald & Barker, 1977, 1978; Chow & Mathers, 1986). A decrease in average channel duration in the presence of penicillin has been reported (Chow & Mathers, 1986) and was consistent with a channel block mechanism and with the findings of this study. Since the open state time constants were decreased in a concentration-dependent fashion by penicillin, this suggested that penicillin was producing open channel blockade rather than an allosteric regulation of the GABA receptor channel. However, these data also suggested that the blockade of the channel was dependent on the kinetic conformational state of the receptor. Results from computer simulation showed that simple open channel block with a single blocker association rate for each of the three open conformation states of the channel could not explain the data. Computer simulations facilitated understanding of the interaction of a putative blocker in a multi-state kinetic reaction scheme with the limitations of measurement resolution. Results of simulations best approximated the experimental data if penicillin association and dissociation rates were different for each open state. When the blocking mechanisms for each open state were considered independently, the microscopic kinetics were consistent with a simple open channel block mechanism for each open state.

#### *Single channel closed properties*

The closed properties of single channel GABA receptor currents also varied with penicillin concentration. The distributions of closed durations were complex. Computer simulations indicated that this complexity was likely due to unequal penicillin unblocking rates for each of the open states and due to limited resolution of the recording system. Since it was not possible to determine the exact number of channels in a patch, analysis was primarily limited to closures less than 100 ms, and thus, closures were more likely to be from a single receptor channel. However, since care was taken to include only recordings from patches which had minimal

occurrence of multiple openings, and at  $2\ \mu\text{M}$ -GABA receptor channels had an open probability (per cent time open) of about 2–4%, it is likely that recordings were obtained from patches containing only one to three active channels.

The brief intraburst gaps found in the absence of penicillin and the blocked periods produced by penicillin appeared to be independent. In this laboratory, the brief intraburst time constants (0.2 and 2 ms) have been shown to be unaltered in those currents evoked by low concentrations of GABA (Macdonald *et al.* 1989*a*) or muscimol (R. E. Twyman & R. L. Macdonald, unpublished), enhanced by barbiturates (Macdonald *et al.* 1989*b*), benzodiazepines (Rogers, Twyman & Macdonald, unpublished) or steroids (R. E. Twyman & R. L. Macdonald, unpublished), or reduced by bicuculline, picrotoxin, pentylenetetrazol or  $\beta$ -carboline (authors' unpublished results). It has been suggested that these brief gaps represent brief blocks or intrinsic closures of the channel and are independent of the gating mechanism for channel closure (Twyman *et al.* 1990). The independence of these closures with GABA or muscimol concentration and preliminary results studying anions such as chloride or HEPES suggest that they represent intrinsic closures of the channel. At low penicillin concentrations, the closed duration time constants associated with these states were well resolved, suggesting that the mechanism responsible for these closures were not affected by penicillin. If these closures were intrinsic to the channel pore, penicillin must bind at a physical site removed from the domain responsible for them even though kinetically, the penicillin blocked state and these closed states were closely associated.

#### *Single channel burst properties*

Finally, the burst properties of single GABA receptor currents were penicillin concentration-dependent. The results revealed prolongation of average burst durations in the presence of penicillin consistent with an open channel block mechanism. With ionophoretically applied GABA to spinal cord neurones in culture, penicillin appeared to consistently prolong the decay phase of GABA-evoked currents (Macdonald & Barker, 1977, 1978). The observed prolonged decay of GABA current can be explained by the prolongation of GABA-evoked bursts through blockade of open channels by penicillin.

#### *Penicillin mechanism of action at the GABA receptor*

The data presented in this study were most consistent with an open channel block mechanism. However, the decrease in open duration time constants may have been produced by an allosteric regulatory mechanism that increased exit rate(s) out of the open states. An increased transition rate to the brief closed states could be eliminated because the proportions of their exponential components did not increase with penicillin concentration. Also, an allosteric increase in the remaining transition rates (1–12, 2–11, 3–10), would have produced a penicillin concentration-dependent decrease in the measured burst durations. Computer simulations supported both of these suppositions. Therefore, for this model of the GABA receptor, allosteric modulation of the open state exit rates by penicillin seemed unlikely.

These results suggested that the accessibility of penicillin to a single binding site

within the channel was dependent upon the kinetic state or open conformation of the channel. Blockade of the receptor when it opened to a brief open state was different compared to when it opened to the longer open states suggesting a physical or conformational change in the accessibility of penicillin to its binding site. To speculate on a physical mechanism, opening of the channel exposes a binding pocket and penicillin would bind more readily and avidly to the channel when it was in the unstable open conformations, whereas if the channel opened to the more stable open conformations, penicillin binding would be more difficult and less tight.

How many penicillin molecules were required for block? Log-log plots (not illustrated) of the total current evoked by GABA as a function of penicillin concentration (100–5000  $\mu\text{M}$ ), revealed a slope of  $-0.4$ . This slope suggests that perhaps only one penicillin molecule was necessary to block the open states of the GABA receptor channel and implies that only one accessible binding site for penicillin was present on the GABA receptor.

In these outside-out patches in symmetrical chloride solutions and voltage clamped at  $-75$  mV, chloride ion flow was from the inside to the outside of the patch and blockade was observed when penicillin was applied from the outside of the patch. In these solutions at pH 7.4, penicillin was negatively charged ( $\text{pK} = 2.76$ ), and therefore, increased blockade at positive patch potentials was expected for the anionic GABA receptor channel in symmetrical chloride solutions. Indeed, penicillin blockade of the receptor channel was more effective at positive patch potentials. Given this voltage sensitivity and that the penicillin molecule is large ( $\text{MW} = 350$ ), the penicillin binding pocket must be close to the outer surface of the receptor channel.

The results of this study were most consistent with a mechanism that penicillin produced channel blockade of the GABA receptor through a simple open channel block of *each* of the GABA receptor open state conformations. The results were consistent with association and dissociation rates of the blocker that were related to the kinetic state of the receptor and suggested that the blocking mechanism was dependent on the active structural conformation of the open channel.

## APPENDIX

State transition rates for simulated models (Scheme 1)

States (i,j)	Transition rate	States (i,j)	Transition rate
0 11	90	11 0	4
1 4	435	4 1	5500
1 5	215	5 1	750
1 12	910	12 1	35
2 6	196	6 2	5500
2 7	97.5	7 2	750
2 11	167.5	11 2	90
3 8	90	8 3	5500
3 9	45	9 3	750
3 10	45	10 3	35
10 11	50	11 10	40
11 12	50	12 11	10000000
12 <sup>1</sup> 13	25	13 12	20000000

Additional transition rates for blocked models (Scheme 2)

## APPENDIX (cont.)

## Scheme 2, Blocked

States (i,j)		Model 1	Model 2	Model 3
		Transition rate	Transition rate	Transition rate
14	1	100	100	55
15	2	100	100	75
16	3	100	100	105
1	14	550000	2110000	2110000
2	15	550000	690000	690000
3	16	550000	275000	275000

Transition rates are  $s^{-1}$  except for agonist or blocker binding transitions (12-11, 13-12, 1-14, 2-15 and 3-16) which are  $M^{-1} s^{-1}$ .

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