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SINGLE CHANNELS ACTIVATED BY HIGH CONCENTRATIONS OF GABA IN SUPERIOR CERVICAL GANGLION NEURONES OF THE RAT

BY CLAIRE F. NEWLAND, DAVID COLQUHOUN AND STUART G. CULL-CANDY

From the MRC Receptor Mechanisms Group, Department of Pharmacology, University College London, Gower Street, London, WC1E 6BT

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SUMMARY

1. Single-channel currents evoked by high concentrations of GABA (10–2000 μM) have been analysed to investigate the characteristics of $GABA_A$ receptor channels in outside-out patches from rat sympathetic neurones. When high concentrations of GABA were applied to a patch, channel openings occurred in prolonged clusters (3·8 ± 3·7 s (mean ± S.D.) at 50 μM-GABA) consisting, on average, of 350 apparent openings per cluster. Individual clusters were separated by long silent intervals.

2. Channel openings were to many (often ill-defined) conductance states (range 7–36 pS), but the most frequently observed conductance level was approximately 30 pS, (29·6 ± 0·34 pS). Only these clusters during which the channel was open to this main state conductance for at least 95% of the cluster open time were used in the analysis of probability of being open. Other less frequently observed conductance levels were 15–18 and 22–23 pS, while levels of 33–36 and 7–9 pS were occasionally, but reliably, observed.

3. Bursts within clusters were defined as a series of openings separated by closed intervals shorter than some critical value, t_c. At 50 μM-GABA the mean burst length was 439 ± 434 ms (± S.D., t_c = 50 ms).

4. The probability of being open, p_o, during bursts within clusters has been analysed as a function of GABA concentration. As expected, increasing the concentration of GABA resulted in an overall increase in p_o. However, for a given agonist concentration there was a wide spread in p_o, far greater than that predicted for a population of identical and independent receptor channels (demonstrated by comparison with simulated channel activity).

5. The wide range of p_o values at a particular concentration of GABA was not due to inappropriate selection of t_c. At 50 μM-GABA the range of p_o values was similar for t_c of 20–1000 ms, although the overall mean p_o became lower (0·64 rather than 0·81).

6. On the basis of simulated channel activity, it appears that most clusters which do not contain multiple openings, arise from the activity of one individual channel. Furthermore, there was no detectable tendency for gaps between bursts to be shorter in the middle of a cluster than at its ends. Therefore it is unlikely that variability in p_o arose from overlapping activity of two or more channels.

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7. The values for p_o, mean open time, and mean shut time for bursts within the same cluster, and between different clusters, were compared by a randomization test. This was to determine whether the wide variability in p_o could plausibly occur if all the open and closed intervals came from the same population. Values of p_o were found to differ not only between one channel and another (i.e. between clusters), but also for the same channel at different times (i.e. between bursts within a cluster).

8. The heterogeneity of GABA receptor channel properties results, at least in part, from one type of receptor channel that changes its activity with time. However, heterogeneity resulting from the presence of several subtypes of GABA receptor channels cannot be excluded.

INTRODUCTION

Studies on the kinetics of $GABA_A$ receptor channels in various vertebrate neurones (by the use of patch-clamp techniques) have employed only low concentrations of agonist. The most recent of these reports suggest a single population of GABA receptor channels with complex gating (Mathers, 1985; Mathers & Wang, 1988; Macdonald, Rogers & Twyman, 1989; Weiss & Magleby, 1989). For other agonist-gated ion channels, such as the muscle nicotinic acetylcholine receptor channel, kinetic analysis of single-channel currents activated by high concentrations of agonist has provided additional and complementary information to that obtained at low agonist concentrations (e.g. Sine & Steinbach, 1984*a*, 1987; Ogden & Colquhoun, 1985; Auerbach & Lingle, 1986, 1987; Colquhoun & Ogden, 1988). There are, however, no published studies of single-channel currents activated by high concentrations of GABA. This approach has revealed heterogeneous behaviour of the GABA receptor channels in mammalian neurones that would not have been apparent at low agonist concentrations.

METHODS

Preparation of dissociated cells

Superior cervical ganglion neurones were dissociated from 17-day-old female Sprague–Dawley rats as previously described (Cull-Candy, Magnus & Mathie, 1986; Cull-Candy & Mathie, 1986) with some modifications. Briefly, superior cervical ganglia were removed from rats killed by chloroform anaesthesia; ganglia were cleaned of fat, and each ganglion cut into three pieces. The pieces were incubated and agitated for 15 min at 37 °C in 10 ml HEPES-buffered MEM (Eagle's Modified Minimum Essential Medium; Flow), containing 266 U ml^{-1} collagenase (Worthington Biochemical Corporation). Following centrifugation at 200 *g* for 1 min, the ganglia were incubated and continually agitated for a further 30 min at 37 °C in 10 ml MEM containing 6 mg ml^{-1} bovine serum albumin (Sigma), 11000 U ml^{-1} trypsin (Sigma, type X11-S), and 3 μg ml^{-1} deoxyribonuclease I (Sigma, type IV). The enzyme reaction was stopped by addition of medium containing rat serum (2% by volume), and the ganglion pieces were mechanically dissociated by repeated passage through a flamed-down Pasteur pipette. The resulting cell suspension was spun through horse serum (at 200 *g* for 5 min) to produce a pellet that was resuspended in a medium containing fetal calf serum (2%, complement inactivated; Sigma), rat serum (2%), $NaHCO_3$ (0·131% by weight), D-glucose (1·05% by weight), NGF-7s (0·025 μg ml^{-1}; Sigma), L-glutamine (2 mM; Sigma), penicillin (50 u ml^{-1}; Sigma), streptomycin (50 u ml^{-1}; Sigma), insulin transferrin sodium selenite (1 μg ml^{-1}; Sigma), 199 low-bicarbonate Earle's Modified salts (10% by volume; Flow). The cells was filtered through a 50 μm mesh and plated on air-dried collagen-coated cover-slips in multiwell plates (Nunc) at a density of 1·5 ganglia per well. These were then maintained at 37 °C in a water-saturated atmosphere of 95% air and 5% CO_2.

Electrophysiological recording

For electrophysiological experiments cells were usually used within 5–6 h or plating, although in some experiments they were used up to 48 h after dissociation. Single-channel currents were recorded from outside-out patches obtained from the cell soma, by means of conventional patch-clamp methods (Hamill, Marty, Neher, Sakmann & Sigworth, 1981). The cells were viewed under phase contrast with a ×40 water immersion objective (total magnification ×640). The cells and patches were bathed in an extracellular solution containing (mM): NaCl, 150; KCl, 2·8; $CaCl_2$, 1·0; Na-HEPES, 10; $MgCl_2$, 2; pH 7·2. The extracellular solution (also containing GABA, where indicated) was applied to the patch or whole-cell via a two-way Hamilton tap connected to a catheter that was carefully positioned under the water-immersion lens. Total exchange of solution in the bath (judging from the 10–90% rise time of changes in pipette tip potential when the bath solution was changed from one containing 70 mM-NaCl to one containing 150 mM-NaCl) usually took approximately 1–4 s. When a cell was directly in the path of the outlet tube, the solution change around the cell took 300–400 ms (judged from the speed of development of whole-cell currents).

Patch pipettes were filled with an internal solution which contained (mM); CsCl, 140; $CaCl_2$, 1·0; K-HEPES, 10; K-EGTA, 10; pH 7·2. Patch-pipettes were pulled from thick-walled borosilicate glass containing glass fibres (o.d. 1·5 mm and i.d. 0·86 mm, Clark Electromedical). Pipettes were Sylgard coated and heat polished, and when filled with the internal solution used for the recording they usually had resistances of 5–15 MΩ. Seal resistances of at least 100 GΩ were routinely obtained. Single-channel currents were recorded with an Axopatch 1B amplifier (Axon Instruments Inc.; internal filter set at 10 kHz), and put on FM tape (Racal Store 4; 5 kHz −3 dB). All experiments were performed in a temperature-controlled room (21–23 °C).

Analysis of single-channel currents

Replayed single-channel currents were filtered at 1·5–2·5 kHz (−3 dB Barr & Stroud, 8-pole Bessel type) and digitized continuously at 15–25 kHz (i.e. ten times the filter cut-off frequency). Single-channel transitions were detected by a 50% threshold-crossing program; a resolution of 1·5 × the filter rise time was subsequently imposed on the results (Colquhoun & Sigworth, 1983), giving resolutions of 200–300 μs. The threshold-crossing method was considered adequate because it was not necessary, for the purposes of defining bursts of openings, to have a very high resolution. Furthermore, the exceptionally large amount of open channel noise made the use of time course fitting impractical.

Shut time, open time and burst length distributions. Analysis was restricted to those runs of single-channel currents during which the channel opened mostly to the approximately 30 pS conductance level. Any openings to other conductance levels were excluded, as were the shut periods before and after the opening. On the rare occasions when two or more channels opened simultaneously the whole event was disregarded, as were the closed intervals either side of it. Distributions of shut times were fitted with probability density functions using the method of maximum likelihood (Colquhoun & Sigworth, 1983). Bursts were defined as a series of openings separated by closed intervals shorter than some critical value, t_c. The values of t_c were determined from shut time distributions using the method of equal percentage of misclassified bursts (Colquhoun & Sakmann, 1985), the two shortest shut time components being defined as intraburst closed times. For display purposes, all histograms show the distribution of log interval, with the ordinate on a square root scale (McManus, Blatz & Magleby, 1987; Sigworth & Sine, 1987). Therefore each exponential appears as a peaked function, the maximum of which corresponds to the time constant of the exponential.

Probability of being open. The probability of being open, p_o, for bursts was calculated by numerical integration of the digitized record, according to

$$p_o = \frac{\text{integral}}{\text{burst length} \times \text{mean amplitude}}$$

where the mean amplitude for each cluster was determined from point amplitude histograms (see below). This allowed for any small changes in single-channel current with temperature (or other factors), that may have occurred during the experiment. For each cluster the baseline was

estimated by taking the mean of two sections of the digitized current record, one measured immediately before the start of the cluster, the other immediately after its end. This will compensate for any linear drift. In addition baseline drift was checked by visual inspection of current records with the computed baseline superimposed on it. This method of calculating p_o is less dependent on the resolution of the system than calculation of p_o from open and shut times, because any linear filter will not change the area under the observed current trace.

Amplitude distributions. The exceptionally large open channel noise (compared with the shut channel noise) of the GABA-evoked channels in these cells made fitting of channel amplitudes with cursors impracticable. Therefore, in most cases, the amplitude levels of single-channel currents were obtained from distributions of the amplitudes of individual data points (with subtraction of the mean shut channel level). These are referred to as 'point amplitude histograms'. In most cases points for the histogram were selected from the record during periods when the channel was shut, or from periods while it was 'open' (i.e. without detectable shut periods) so that most of the points representing transitions between open and shut levels were excluded. Histograms were fitted with a single Gaussian, or with the sum of several Gaussians, by the method of maximum likelihood (Colquhoun & Sigworth, 1983); reasonable-looking fits were usually obtained despite the fact that one would not, in principle, expect the distributions (for open points at least) to be exactly Gaussian.

The possible presence of *discrete* sublevels was further investigated by inspection of the distribution of the mean amplitudes of short stretches of data that had low variance, and which therefore represented sojourns at a fairly constant amplitude level (using the 'sublevel detection' method of Patlak, 1988). The variance and mean amplitude were calculated for sections of ten points, successive sections being taken by advancing along the digitized record by one point each time. All of those sections for which the standard deviation was greater than some specific multiple (usually 1·0) of the baseline root mean square noise was eliminated, and the histograms were constructed from the mean amplitudes of sections not so eliminated. These are referred to as 'mean low-variance amplitude histograms'.

Single-channel current amplitudes are mostly quoted as chord conductances, assuming a linear current–voltage relationship and a reversal potential of 0 mV. The reversal potential estimated from whole-cell and single-channel current–voltage relationships ranged from 0 to +3 mV, (the theoretical value, estimated from the chloride concentrations, rather than chloride activities, is −2·8 mV). Point amplitude and low-variance amplitude histograms gave some idea of whether or not there were significant contributions from levels other than the approximately 30 pS level during a cluster.

Randomization test. Bursts within the cluster, and bursts from different clusters, were compared by a randomization test (Patlak, Ortiz & Horn, 1986) in order to determine whether the open times and shut times occurring during different bursts all came from the same distributions of open and shut intervals. This test was used to compare the values of p_o, mean open time and mean shut time, for bursts of at least 200 ms duration (more than forty openings) and at the same agonist concentration. Briefly, the observed scatter (referred to as observed S) for the bursts was calculated

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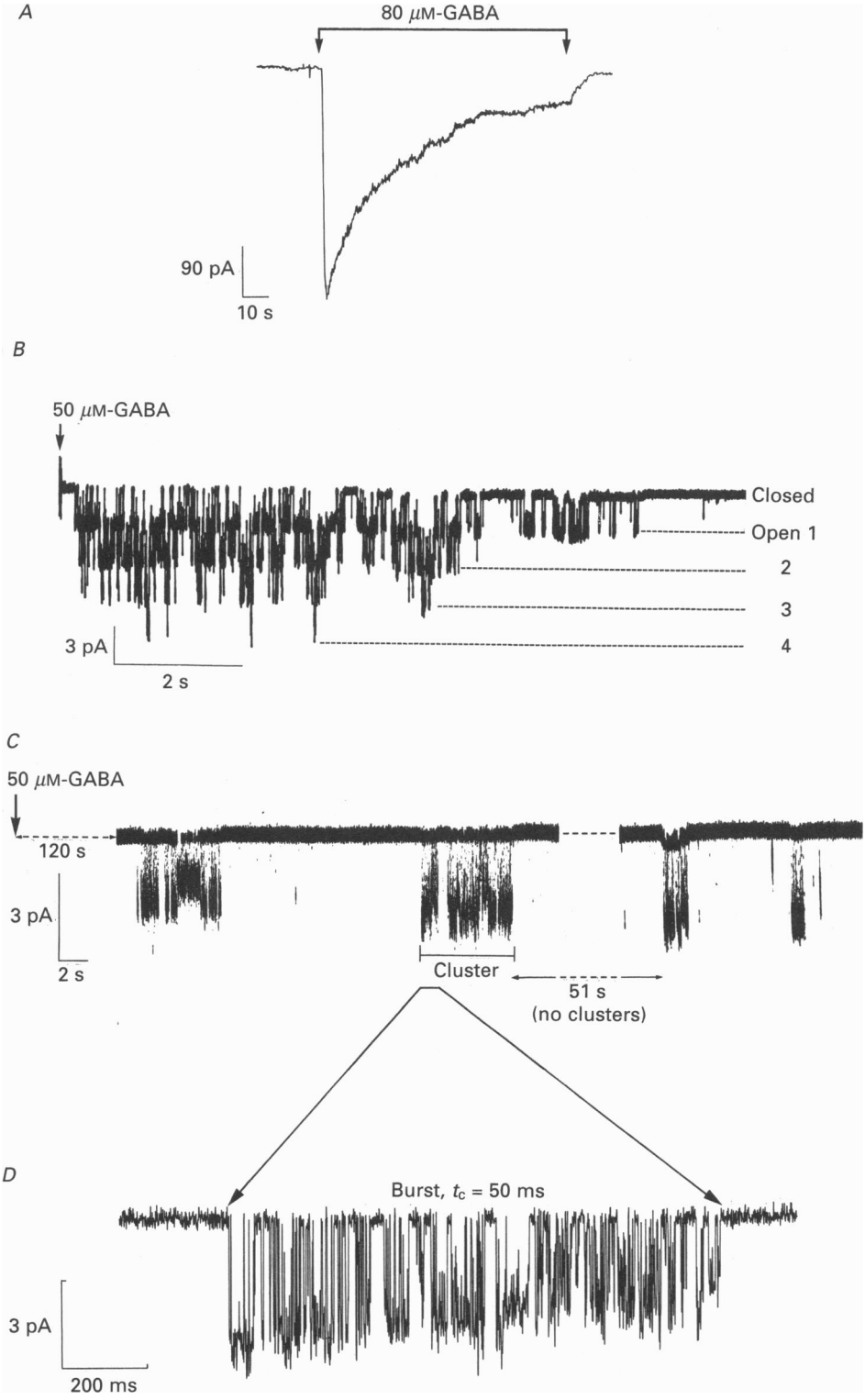
Fig. 1. Desensitization of GABA-responses in whole-cell and outside-out patches. *A*, whole-cell current response of a cell held at −60 mV, showing desensitization in the continued presence of 80 μM-GABA. Calibration, 90 pA and 10 s. *B*, application of 50 μM-GABA (drug perfusion starts at the beginning of the trace) to an outside-out patch, causing simultaneous opening of up to four channels. The number of open channels are indicated on the right-hand side. After 5–10 s in the continued presence of 50 μM-GABA only one channel is active at a time. Calibration, 3 pA and 2 s (V_m = −80 mV). *C*, examples of 'desensitization clusters' of openings produced by 50 μM-GABA. The four consecutive clusters were recorded in the continued presence of GABA and approximately 120 s after the initial GABA application. Individual clusters are separated by long silent intervals of 7–51 s (V_m = −100 mV). Calibration, 3 pA and 2 s. *D*, an example of a single burst shown on an expanded time base. As indicated, this burst is the first one in the second cluster in trace *C*, and exceeds 1 s in duration (defined by a t_c of 50 ms). Note that the effective filtering of the plot is 890 Hz (−3 dB). Calibrations, 3 pA and 200 ms. Temperature, 22 °C.


A
80 μM-GABA
90 pA
10 s
B
50 μM-GABA
Closed
Open 1
2
3
4
3 pA
2 s
C
50 μM-GABA
120 s
3 pA
2 s
Cluster
51 s
(no clusters)
D
Burst, t_c = 50 ms
3 pA
200 ms

according to

$$S = \sum_{i=1}^{N} n_i(\bar{y}_i - \bar{\bar{y}})^2,$$

where N is the number of bursts compared, n_i is the number of openings in the ith burst, $\bar{y}_i$ is the mean open time (*or* p_o, *or* mean shut time) for the ith the burst, and $\bar{\bar{y}}$ is the overall mean open time (*or* overall p_o, *or* overall mean shut time). Histograms of randomized scatter (referred to as randomized S) were obtained under the null hypothesis that bursts are homogeneous, by generating 1000 or more sets of N bursts, as follows. Sets of artificial bursts, containing $n_1, n_2, \ldots, n_N$ openings, were generated by a random selection of open and shut times from the total population of observed intervals. Each artificial burst consisted of the same number of openings as the observed bursts. A value of randomized S was calculated from the equation above, for each set of N bursts generated in this way. If the randomized S values exceed the observed S value only rarely (in a proportion P, say, of cases), this implies that the open time (*or* p_o, *or* shut time) genuinely differ between bursts.

RESULTS

Basic features of responses to high concentrations of GABA

Whole-cell current responses to GABA showed obvious desensitization at concentrations above 10 μM. Figure 1*A* shows a typical whole-cell response to 80 μM-GABA in a superior cervical ganglion neurone held at -60 mV. The current was reduced to approximately 15% of the peak amplitude within 90 s. Desensitization was also apparent in the single-channel records, as illustrated in Fig. 1*B*. The initial application of a desensitizing concentration of GABA (50 μM) to an outside-out patch resulted in the simultaneous opening of several channels (up to four, in this patch). However, in the continued presence of GABA the receptors desensitized, such that apparently only one channel was active at a time. In the example illustrated this occurred within about 7 s of GABA application. Under these conditions the other receptors had entered long-lived desensitized states. GABA-like openings were only rarely seen prior to the application of GABA (see Mathers, 1985) to outside-out patches, and were of the order of 1 ms. In a desensitized outside-out patch such as the one depicted in Fig. 1*C*, the openings occur in prolonged clusters each of which can last several seconds, and these clusters are separated by long silent intervals (1–480 s). For example, the clusters illustrated in Fig. 1*C* were separated by shut periods of 7, 14 and 51 s. At 50 μM-GABA the mean cluster length was $3{\cdot}8 \pm 3{\cdot}7$ s (mean $\pm$ S.D., $n = 19$ clusters) for clusters which opened mainly to the 30 pS conductance level. Cluster lengths for clusters which opened mainly to other conductance states were not systematically measured, but were not obviously different from the lengths of the 30 pS clusters.

In the present study, analysis of the probability of being open was restricted to those clusters that opened mostly to a 30 pS level, since this was the most frequently occurring conductance state. Bursts within clusters were defined as a series of openings separated by closed intervals shorter than a critical value, t_c (see Methods). A typical burst from within one of the clusters in Fig. 1*C*, is illustrated on an expanded time base in Fig. 1*D*. At 50 μM-GABA the mean bust length ($\pm$S.D.) was 439 ± 434 ms ($n = 40$, $t_c = 50$ ms), and the apparent number of openings per cluster was about 350. The apparent mean open time and mean shut time (for gaps within bursts) were 8·9 ms and 3·66 ms respectively (at 50 μM-GABA).

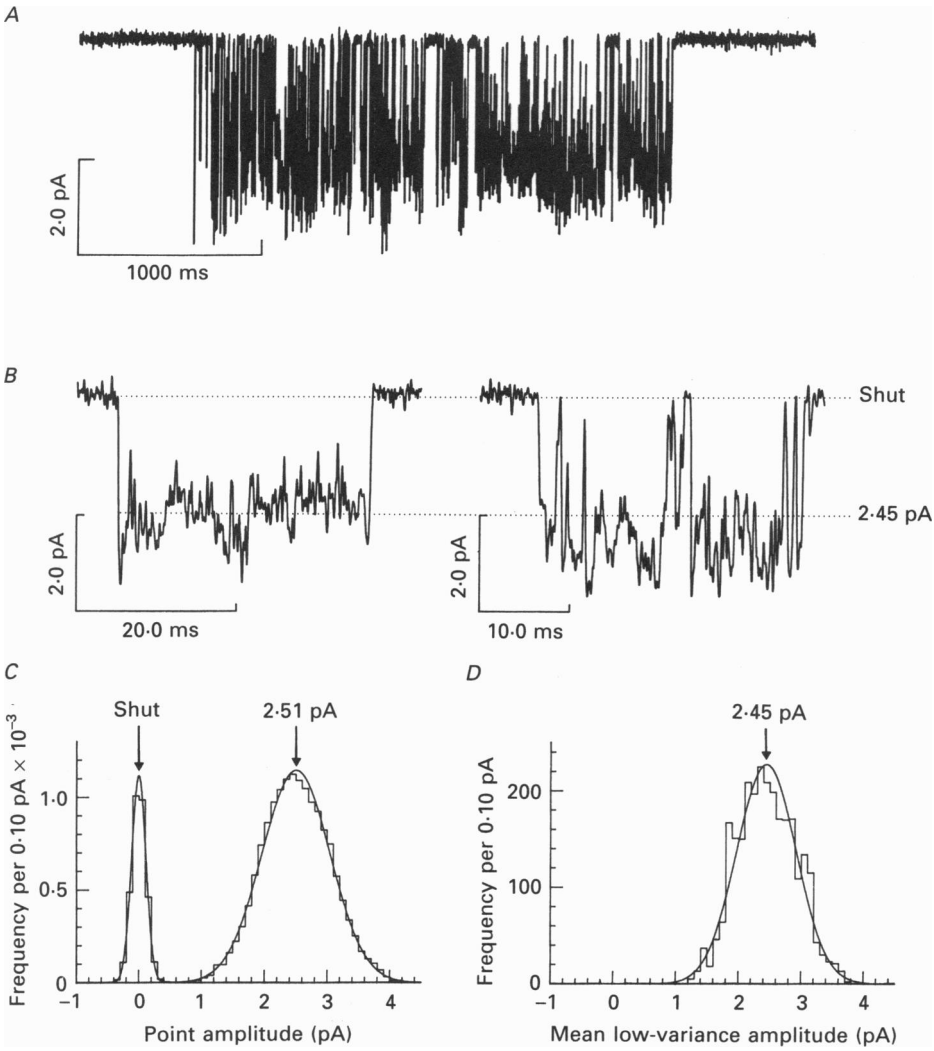


Fig. 2. *A*, a selected record to illustrate repeated transitions between multiple-conductance levels occurring during a single cluster (50 μM-GABA, outside-out patch, $V_m = -120$ mV). Long shut periods, > 56 s, occurred on either side of the cluster (not shown). Calibration, 2 pA and 1 s. *B*, sections of the cluster in *A*, on an expanded time scale, to illustrate what appear to be transitions between discrete conductance states. The dotted lines indicate the shut level and the mean open level of 2·45 pA (equivalent to 20·4 pS), as obtained from the low-variance amplitude distribution (*D*). Calibration, 2 pA and 10 ms; records filtered at 2 kHz, −3 dB. *C*, amplitude histograms for open and shut points during the cluster. Each histogram was fitted with a single Gaussian; mean open point amplitude of 2·51 pA, (equivalent to 20·9 pS). Note that multiple-conductance states were not discernible as discrete peaks from the point amplitude distribution, although the width of the open point amplitude distribution (S.D. of 0·54 pA) was far greater than that of the shut point distribution (S.D. of 0·11 pA). Currents used for the histogram were filtered at 2 kHz, −3 dB. Frequency scale refers to the open point histogram. *D*, mean low-variance amplitude histogram for the open states of the cluster in *A*. The histogram was fitted with a single Gaussian, mean open level 2·45 pA (equivalent to 20·4 pS). Currents used for the histogram were filtered at 2 kHz, −3 dB. Temperature, 22 °C.

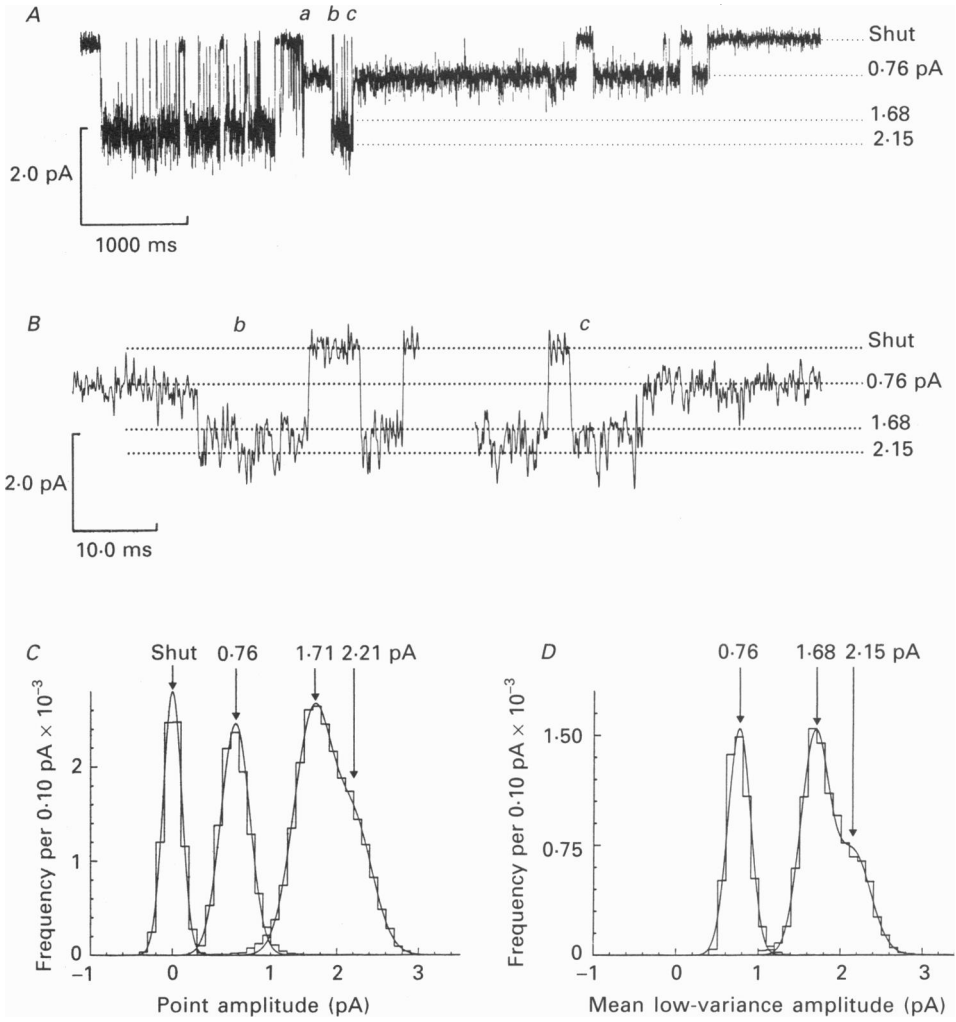


Fig. 3. *A*, a single cluster selected to illustrate switching between high-conductance and low-conductance levels. The low level is about 0·76 pA and the high level may actually consist of two rather closely spaced levels (values of 1·68 and 2·15 pA are suggested by the sublevel detection plot in *D*). These levels are indicated by dotted lines (outside-out patch, 2 mM-GABA, $V_m = -100$ mV). Long shut periods occurred on either side of this cluster. At points *a*, *b* and *c* during the cluster, the channel switched between distinct conductance states (7·6 pS, 17–22 pS and closed). Calibration, 2 pA and 1 s. *B*, section *b* and *c* of the cluster in *A*, shown on an expanded time scale. The dotted lines indicate the shut level and the conductance levels of 7·6, 16·8 and 21·5 pS. Calibration, 2 pA and 10 ms; (filter, 2·5 kHz, −3 dB). *C*, three point amplitude histograms for the cluster shown in *A*. These were determined separately from sections of the record in which the channel was shut, in which it was open at the lower level, and in which it was open at the higher level. Single Gaussians were separately fitted to the shut point amplitude histogram and to the left-hand open point amplitude distribution (mean amplitude, 0·76 pA). The sum of two Gaussians was fitted to the right-hand open point amplitude distribution (mean amplitudes, 1·71 an 2·21 pA). The frequency scale refers to the right-hand distribution (maximum frequency of the 0·76 pA distribution was 11000 per 0·1 pA). Current record filtered at 2·5 kHz, −3 dB. *D*, mean low-variance amplitude distributions of the open states of the cluster in *A*. The left-hand distribution was fitted with a single Gaussian

Multiple-conductance states

Many different conductance levels of the GABA receptor channel ranging from 7–36 pS were observed in all patches and at all GABA concentrations (10–2000 μM). Unlike multiple-conductance GABA channels in spinal neurones (Bormann, Hamill & Sakmann, 1987) these conductance states were often difficult to resolve, apparently because the levels were too close together and too short lived, and because of the exceptionally large open channel noise (relative to the shut channel noise). In terms of their conductances, clusters of channel openings fell into three general categories: (1) within a single cluster the channel appeared to 'wobble' between may different conductance levels, (2) there were a few clear, large changes in conductance within a cluster, which were obvious from open point amplitude and mean low-variance amplitude histograms, and (3) there were whole clusters with fairly consistent levels, that clearly differed from one cluster to another in the same patch. Figures 2, 3 and 4 illustrate examples of each of these categories of channel openings within clusters.

The records in Fig. 2*A* and *B* show single-channel openings that appear to contain many different ill-defined conductance levels (none of which correspond to the overall mean open channel amplitude of 2·45 pA determined from the mean low-variance amplitude histogram of this cluster in Fig. 2*D*). It is also clear from Fig. 2*B* that the current during an individual opening was usually much noisier than the baseline (i.e. the shut channel).

Discrete multiple-conductance levels could not be resolved as discrete peaks from the open point amplitude distribution in Fig. 2*C*. However, the presence of a wide range of conductance levels was reflected by the much larger standard deviation (S.D.) of the open point amplitude distribution, compared with the S.D. of the shut point amplitude distribution, both of which could be fitted reasonably well with single Gaussian functions. In Fig. 2*C* the S.D. of the open and shut point amplitude distributions are 0·54 and 0·11 pA respectively, so the excess open channel noise variance is 23-fold greater than the shut channel variance. The mean single-channel conductance from the histogram is 21 pS. Multiple-conductance peaks were also not detectable in the mean low-variance amplitude histogram obtained from the same data (Fig. 2*D*). This histogram consisted of the mean amplitudes of sections of digitized current record (ten points) that had a variance as low as that of the shut channel. A single Gaussian has been fitted to the distribution; although there is some sign of multiple peaks these were not consistent from one cluster to another.

On the other hand, amplitudes of various conductance levels could be clearly resolved when the channel opened to one level for a large proportion or all of the cluster (Figs 3 and 4). Figure 3*A* shows an example of a cluster that opened initially to a current level (or levels) of 1·68–2·15 pA. After approximately 1·8 s the current dropped to 0·76 pA; it returned briefly to the higher level, and then continued at 0·76 pA for a further 3·5 s. Figure 3*B* illustrates direct transitions between current levels of 0·76 pA and the higher level (1·68–2·15 pA) on a faster sweep speed. The presence of such transitions and of direct closures from the higher level, indicate that

(mean amplitude, 0·76 pA); the sum of two Gaussians was fitted to the right-hand histogram (mean amplitudes, 1·68 and 2·15 pA). Current record filtered at 2 kHz (−3 dB). The frequency scale refers to the right-hand distribution (the maximum frequency of the 0·76 pA peak occurred at 11000 per 0·1 pA).

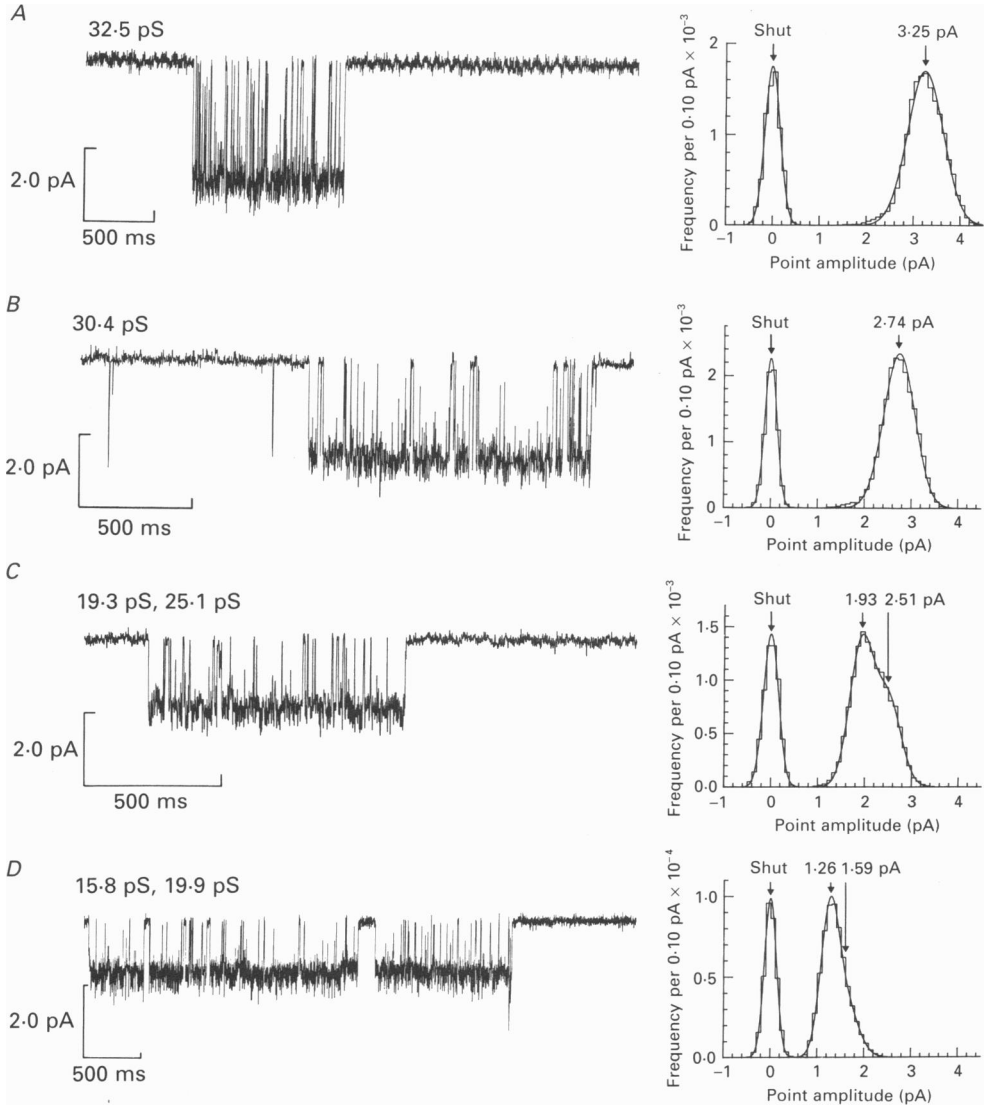


Fig. 4. Selected clusters in which the channel opens to a single conductance level, or to two closely spaced conductance levels. The shut and open point amplitude histograms are shown to the right of their corresponding current records. *A*, cluster recorded from an outside-out patch in 2 mM-GABA ($V_m = -100$ mV). The open point amplitude histogram was fitted with a single Guassian distribution; mean amplitude of 3·25 pA (equivalent to 32·5 pS). *B*, a cluster in a patch exposed to 50 μM-GABA ($V_m = -90$ mV). A single Gaussian was fitted to the corresponding open point amplitude histogram; mean amplitude, 2·74 pA (30·4 pS). *C*, a cluster in the presence of 2 mM-GABA, ($V_m = -100$ mV). The corresponding open point amplitude histogram was fitted with the sum of two Gaussians; mean amplitudes 1·93 and 2·51 pA (19·3 and 25·1 pS; relative areas 0·61 and 0·39 respectively). *D*, a cluster in the presence of 100 μM-GABA ($V_m = -80$ mV). The corresponding open point amplitude histogram was fitted with the sum of two Gaussians; mean amplitudes 1·26 and 1·59 pA (15·8 and 19·9 pS; relative areas 0·62 and 0·38,

these events are due to multiple conductances of a single-channel, rather than superimposed openings from several channels. In Fig. 3*C* and *D* the 0·76 pA level and the higher level are clearly resolved as discrete peaks both from their open point, and mean low-variance amplitude histograms. In both cases the 0·76 pA histogram was compiled from the data separately from the other histograms and fitted with a single Gaussian distribution. It is also clear from Fig. 3*C* and *D* that the higher level may consist of two or more conductance states. The presence of more than one component is clearer in the mean low-variance amplitude plot (Fig. 3*D*) than in the open point amplitude plot (Fig. 3*C*). Both are fitted reasonably well by the sum of two Gaussians, and the estimates of the two discrete higher levels (1·68 and 2·15 pA from the former and 1·71 and 2·21 pA from the latter) agree well, especially in view of the fact that the distributions are not, in principle, expected to be described exactly by a sum of Gaussians. However, it can be seen that these current levels, drawn in Fig. 3*B* (1·68 and 2·15 pA), correspond only roughly with discrete levels in the data, so it may well be that the description of three *discrete* levels is an oversimplification. The current levels suggested by the histograms correspond to multiple-conductance levels of 7·6, 17 and 22 pS.

Figure 4*A–D* illustrates four representative clusters (from different patches), during which the GABA channel opened to only one level, or to two closely spaced conductance levels. The clusters in Fig. 4*A* and *B* appeared to open mainly to one distinguishable level, since the open point amplitude histogram of each of these clusters was adequately fitted by a single Gaussian function (though, as always, the S.D. was greater than that for the shut point histograms). The estimated conductance levels were 32·5 pS (Fig. 4*A*) and 30·4 pS (Fig. 4*B*) for a reversal potential of 0 mV. Figure 4*C* and *D* shows two clusters each of which appeared to open to at least two closely spaced conductance levels for large proportions of their open time, as judged by their open point amplitude histograms which were fitted with the sum of two Gaussians. The estimated conductance levels for the cluster illustrated in Fig. 4*C* were 19·3 and 25·1 pS (relative areas 0·61 and 0·39, respectively); the estimated conductance levels for the cluster shown in Fig. 4*D* were 15·8 and 19·9 pS (relative areas 0·62 and 0·38, respectively). It was common to see clusters such as these which had fairly consistent amplitudes within a cluster, but clearly different amplitudes from one cluster to another in the same patch.

In summary, the amplitudes of various conductance levels were inferred by fitting of point amplitude, and mean low-variance amplitude, histograms of clusters which fluctuated round one main open level for the whole of the cluster (e.g. Fig. 4*A* and *B*), or which opened to a few conductance levels, but remained at each of these levels for a large proportion of the channel open time (e.g. Fig. 3). Only records where levels could be reliably defined were used. The conductance levels measured from such records were 28·5–30·5, 15–18 and 22–23 pS. Levels of roughly 33–36 and 7–9 pS were reliably, but only occasionally, observed.

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respectively). Calibration for *A–D*, 2 pA and 500 ms. Temperature, 22–23 °C. Shut point amplitude histograms were fitted with single Gaussians (mean 0 pA). The frequency scales refer to the open point amplitude histograms. Channel records low-pass filtered at 1 kHz (−3 dB) for illustrative purposes.

In the present study, the analysis of the probability of being open p_o, as a function of GABA concentration, was restricted to those clusters during which the channel was open to the main state conductance for at least 95% of the (open) time during the cluster (e.g. Fig. 4*B*). The amplitude of the main conductance level was

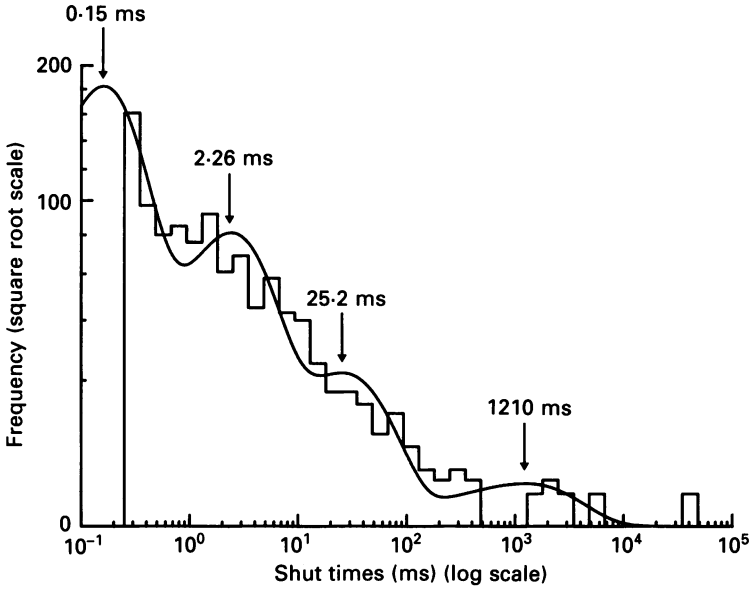


Fig. 5. Frequency histogram of all intracluster shut times, measured from six clusters (from the same patch), which opened mostly to the 30 pS level; patch exposed to 50 μM-GABA; $V_m = -70$ mV; temperature, 22 °C. The histogram shows the distribution of log shut times, with the ordinate on a square root scale, for display purposes. The untransformed shut times between 0·25 to 2000 ms were simultaneously fitted with four exponentials (continuous curve). The time constants were 0·15, 2·26, 25·2 and 1210 ms; relative areas were 0·63, 0·28, 0·08 and 0·006 respectively. Currents filtered at 2 kHz, -3 dB; resolution set at 250 μs. Note that the fit is not very clearly defined.

29·6 $\pm$ 0·34 pS (mean $\pm$ S.E.M., $n = 47$ clusters). The question of whether p_o is the same at the other, less common, conductance levels has not been investigated.

Probability of being open as a function of GABA concentration

Shut time distribution and choice of critical gap length

The aim of the p_o determinations was to define the equilibrium response to specified agonist concentrations of an individual receptor channel, after elimination, as far as possible, of the effects of the desensitization. To achieve this it is necessary to identify sections of the record (bursts) during which one individual channel showed repeated activations without entering a desensitized state (and without interference from the opening of any other channels). The division of the observed record into clusters is quite clear; all the openings within a cluster probably originate from the same channel (see below). It is also likely that the GABA channels are in long-lived desensitized states during silent periods between clusters, as in the case of

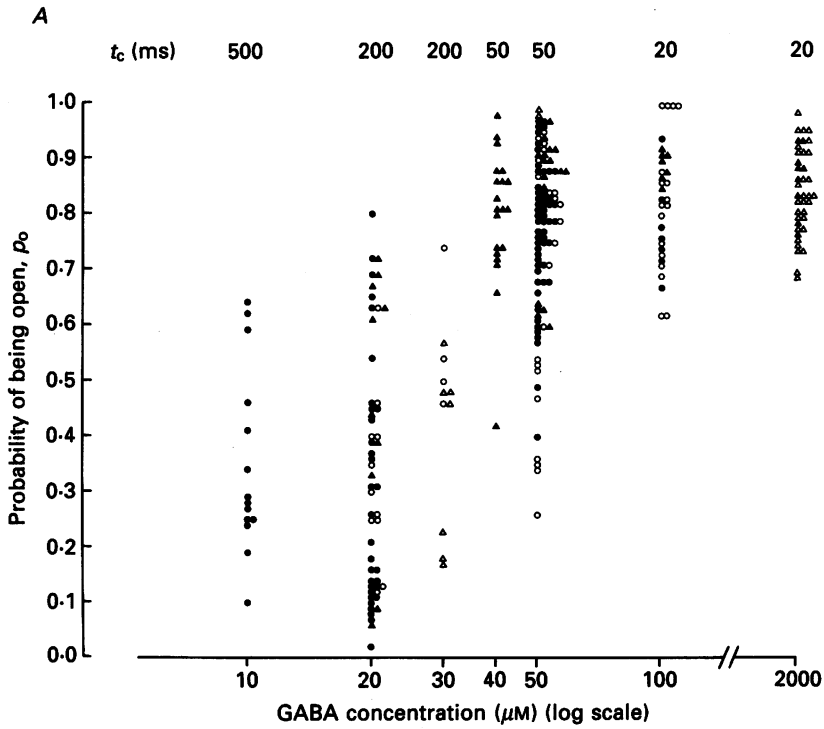
the nicotinic receptor (Sakmann, Patlak & Neher, 1980; Sine & Steinbach, 1987; Colquhoun & Ogden, 1988).

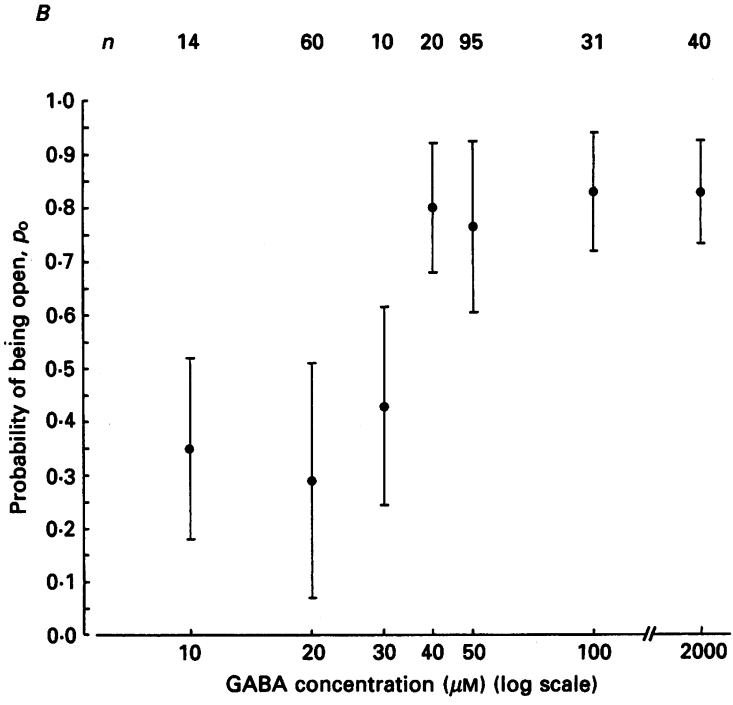
Greater ambiguities arise in trying to decide whether the briefer shut periods within clusters, are spent in (short-lived) desensitized states or whether they are spent in the resting (i.e. activatable) state between activations of a non-desensitized receptor. This problem is, or course, particularly acute at lower agonist concentrations (when intervals between individual activations are long), which is one of the reasons why we have not attempted to give p_o values for GABA concentrations below 10 μM. In the case of the nicotinic receptor there are clear shut periods (gaps) within clusters that are spent in short lived desensitized states (Sakmann *et al.* 1980), and the same is probably also true of GABA receptors. To identify bursts it is therefore necessary to define a critical gap length, t_c, that is short enough to exclude most of these brief desensitized periods, while including most of the gaps between individual channel activations. For the nicotinic receptor it has been suggested (Cachelin & Colquhoun, 1989) that the long gaps between clusters correspond to the microscopic component of desensitization with a time constant of a few seconds, (Katz & Thesleff, 1957; Feltz & Trautmann, 1982) whereas the briefer desensitized periods within clusters correspond to 'ultra-fast' macroscopic desensitization with a time constant of roughly 100 ms (e.g. Brett, Dilger, Adams & Lancaster, 1986). Unfortunately relatively little is known about GABA desensitization, even on the macroscopic scale (although see Akaike, Inoue & Krishtal, 1986; Cash & Subbarao, 1987; Ikemoto, Akaike & Kijima, 1988). In particular, nothing is known about ultra-fast desensitization, so the choice of critical gap length, t_c could not be unambiguous, and must be based largely on inspection of the distributions of intracluster shut times (i.e. shut times within clusters).

Intracluster shut times

Frequency histograms of intracluster shut times were constructed using the intervals measured by the 50% threshold crossing routine (low-pass filtered 2 kHz, −3 dB; filter rise time, 167 μs). These shut times were fitted with probability density functions from 250 μs to 2 s by the method of maximum likelihood (Colquhoun & Sigworth, 1983).

Figure 5 illustrates a typical distribution of the logarithm of intracluster shut times (see McManus *et al.* 1987; Sigworth & Sine, 1987) which were measured from six clusters, all recorded from the same outside-out patch exposed to 50 μM-GABA. In this example, the histogram was fitted with four exponentials, with time constants of 0·15, 2·26, 25·2, and 1210 ms (relative areas 0·63, 0·28, 0·08 and 0·006 respectively). The fit is not very clearly defined, which is not surprising in view of the other evidence, given below, that GABA receptor channels do not show homogenous kinetic behaviour; there may well be more than four time constants. Although the time constants of distributions cannot, in general, be equated with the mean lifetimes of particular states, or set of states, it is necessary to postulate some approximate physical significance in order to proceed. The fastest two components are likely to represent, primarily, spontaneous shut periods within single-channel activations, because we have found similar time constants at lower GABA concentrations, where individual channel activations are better separated: for example with 2–5 μM-GABA


A
t_c (ms) 500 200 200 50 50 20 20
Probability of being open, p_o
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0·0
10 20 30 40 50 100 2000
GABA concentration (μM) (log scale)


B
n 14 60 10 20 95 31 40
Probability of being open, p_o
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0·0
10 20 30 40 50 100 2000
GABA concentration (μM) (log scale)

we find time constants of about 0·25±0·07 and 1·6±0·34 ms (mean±S.D., $n = 7$). Comparable values, for shut periods within single-channel activations, have been found for GABA channels in other central neurones (Mathers, 1985; Macdonald *et al.* 1989; Weiss & Magleby, 1989), and bovine chromaffin cells (Bormann & Clapham, 1985). In addition, similar duration 'within-activation-gaps' have been described for acetylcholine (Colquhoun & Sakmann, 1981; 1985; Sine & Steinbach, 1984*b*, 1986), and glutamate-activated channels (Cull-Candy & Parker, 1982; Howe, Colquhoun & Cull-Candy, 1988).

It is reasonable to suppose that the next slowest time constant primarily represents gaps between channel activations (though at high concentrations these may not be distinguishable from intra-activation gaps). Thus it seems reasonable to use a critical gap length of the order of 100 ms to define bursts with 50 μM-GABA, only the longest intracluster time constant in Fig. 5 being excluded.

Estimates of p_o curves

As expected, increasing the concentration of GABA resulted in an overall increase in the probability of being open, p_o. Figure 6*A* and *B* shows the increase in mean probability of being open during bursts (of at least 100 ms), over the GABA concentration range 10–2000 μM; the t_c value used, and the number of bursts (n) analysed are indicated for each concentration. The results were pooled from different outside-out patches held at −70, −80, −90 and −100 mV, and include a total of 270 bursts. The mean probabilities (±S.D.) of being open (Fig. 6*B*) were 0·35±0·17 (10 μM, $n = 14$ bursts), 0·29±0·22 (20 μM, $n = 53$) 0·43±0·19 (30 μM, $n = 10$), 0·80±0·12 (40 μM, $n = 20$), 0·77±0·16 (50 μM, $n = 95$), 0·83±0·11 (100 μM, $n = 31$), and 0·83±0·1 (2000 μM, $n = 40$). It is apparent from Fig. 6*B* that the maximum mean p_o falls well below 1·0, even at the high concentration of 2 mM-GABA (where the mean p_o value was 0·83). Furthermore, even at these high concentrations, the main state conductance was still approximately 30 pA. There was therefore no evidence to suggest that the GABA molecule was producing a rapid open channel block.

A striking feature of the dependence of p_o on GABA concentration in Fig. 6*A* is the wide range of p_o values observed for any given agonist concentration and any given voltage (−70, −80, −90 or −100 mV). For example, at a concentration of 50 μM-GABA and −70 mV the probability of being open ranged from 0·4 to 0·97 (55

Fig. 6. Dependence of probability of being open, (during bursts within clusters that opened mostly to the 30 pS level) on GABA concentration. *A*, probability of being open as a function of GABA concentration (10–2000 μM). Each point represents a single burst; the numbers of bursts, n, at each concentration, were: 10 μM, $n = 14$; 20 μM, $n = 53$; 30 μM, $n = 10$; 40 μM, $n = 20$; 50 μM, $n = 95$; 100 μM, $n = 31$; 2000 μM, $n = 40$. Results were pooled from five different outside-out patches held at −70 mV (●), −80 mV (○), −90 mV (▲) and −100 mV (△), temperature 22–23 °C. The critical gap length, t_c, used to define the bursts is given above each concentration. Note the wide range of p_o values at each concentration and potential. The concentration axis is not continuous. *B*, mean p_o *vs.* GABA concentration (same data as in *A*). The vertical bars represent ±S.D.; the number of bursts (n) averaged for each point is given at each concentration; the t_c value used to define the bursts is the same as in *A*. Note the large S.D., due to the wide scatter of p_o values, and that p_o increases with increasing GABA concentration up to approximately 40–50 μM.

bursts), and at 20 μM-GABA and -70 mV the range was 0·02–0·80 (53 bursts). Such a wide range of p_o values has not previously been seen for populations of agonist activated channels which are homogeneous (Sakmann *et al.* 1980; Cull-Candy, Miledi & Parker, 1981; Colquhoun & Ogden, 1988).

Expected spread of p_o for a homogeneous population of receptor channels

It is not immediately obvious how much variability would be expected in p_o, from one burst to another, if the receptors behaved in a homogeneous manner. Unfortunately the answer to this question depends on the precise mechanism of action of the receptor channel. Although models have been proposed (e.g. Macdonald *et al.* 1989; Weiss & Magleby, 1989) none has been adequately tested over the entire concentration range (and none has been based on GABA receptors of peripheral neurones). In order to get a rough idea of the sort of variability that might be expected we have, therefore, done simulations based on the simplest mechanisms of channel gating that can produce bursting behaviour, in order to predict the variability of p_o values. These are

$$C_L \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} C_S \underset{\alpha}{\overset{\beta}{\rightleftharpoons}} O, \tag{1}$$

and

$$C_L \underset{\alpha}{\overset{\beta'}{\rightleftharpoons}} O \underset{k_{-B}}{\overset{k_{+B}}{\rightleftharpoons}} C_S, \tag{2}$$

where O represents an open channel, C_L represents a long-lived shut state (entry into which will end a burst) and C_S represents a short-lived shut state. The transition rates (k_{-1}, k_{+1}, β, β', α, k_{+B}, and k_{-B}) shown in both schemes (1) and (2) were selected to give the mean number of openings per burst as approximately 35, the mean open time as 8·9 ms and the mean shut time as 3·66 ms, with the p_o within bursts being 0·72. These are close to the values observed experimentally with 50 μM-GABA, using a t_c of 50 ms. Thus for scheme (1) the transition rates were: for $k_{-1} = 7{\cdot}8\ s^{-1}$; $\beta = 265\ s^{-1}$; $\alpha = 112\ s^{-1}$; $k_{+1} = 0{\cdot}1\ s^{-1}$. For scheme (2) the rates used were: $\alpha = 3{\cdot}2\ s^{-1}$; $\beta' = 0{\cdot}1\ s^{-1}$; $k_{+B} = 109{\cdot}2\ s^{-1}$; $k_{-B} = 273\ s^{-1}$. A random number generator (Wichmann & Hill, 1985) was used to generate simulated records from these models, and the simulated records were divided into bursts using a critical shut time of 100 ms; bursts were well separated because the mean lifetime of C_L was made very long (10^4 ms). Bursts shorter than 100 ms were discounted, as in the analysis of the real experiments, and a histogram of p_o values from the remaining bursts was constructed. The results for both models were (as usual) indistinguishable, so only the results for model (1) are shown in Fig. 7. The histogram of simulated p_o for model (1) is shown in Fig. 7*A*, and the histogram of the observed p_o obtained from the experimental data is shown in Fig. 7*B* (the histograms of the simulated and observed data contain 304 and 87 bursts respectively). Clearly the expected spread of p_o values, for receptors that behave homogeneously, is much less than the observed spread (S.D. of p_o for the simulated and observed bursts were 0·057 and 0·16, respectively). It must be stressed that other mechanisms could make substantially different predictions concerning the expected variability of p_o (see above), but this is the best that can be done at present.

Two experimental problems could have contributed to the variability in p_o, namely, (1) inappropriate selection of t_c, and (2) undetected presence of more than one active channel during a cluster. The possible contribution of these two artifacts will now be considered.

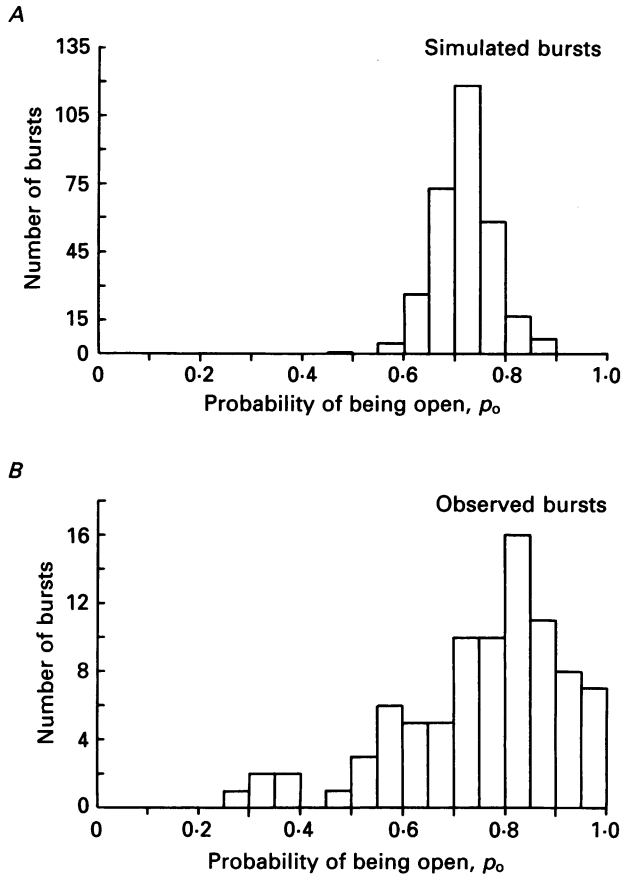


Fig. 7. Comparison of the observed spread of probability of being open, p_o, with that expected for a population of identical and independent receptor channels. *A*, histogram of p_o for 304 bursts of more than 100 ms duration, obtained from a simulated record of channel opening and closing durations, using a t_c of 100 ms (see text for details). Mean p_o was 0·72 *B*, histogram of p_o of eighty-seven observed bursts (50 μM-GABA; t_c = 50 ms; bursts more than 100 ms duration; temperature, 22–23 °C). Mean p_o was 0·76. Note the larger spread of p_o for the observed bursts, compared with the simulated bursts (S.D. of the p_o for the simulated bursts was 0·057, and for the observed bursts was 0·16).

Effect of t_c on the estimation of p_o

Although only one value of t_c (for a given GABA concentration) can be optimal for estimation of the equilibrium concentration–response curve, it seemed desirable to determine, empirically, the spread of p_o values obtained with a range of t_c values. This allowed us to check that the appearance of heterogeneity was not critically dependent on t_c, the optimum value for which was uncertain. Histograms of p_o values

for individual bursts were therefore calculated using a range (20–1000 ms) of critical gap durations (see Auerbach & Lingle, 1986). Figure 8 illustrates the effect of selecting different values for t_c on the spread of p_o, and on the overall mean p_o, of bursts recorded in the presence of 50 μM-GABA.

Each histogram includes data from twenty clusters (at holding potentials of -60 to -100 mV) in the presence of 50 μM-GABA. The bursts within the twenty clusters were defined by t_c values of 20, 50, 100, 200 or 1000 ms (indicated next to corresponding histogram), and only bursts of more than 100 ms duration were included. It is clear that the wide spread in p_o occurs regardless of the value of t_c. This suggests that there is heterogeneity between bursts (see randomization test, below, for further analysis). Reducing the value of t_c obviously increased the number of bursts and reduced the length of each burst. However, at 50 μM-GABA the mean p_o was increased only slightly when t_c was reduced. Thus, the mean p_o was reduced from 0·81 to 0·71 when t_c was increased from 20 to 200 ms. The change may reflect the inclusion of some of the brief desensitization gaps with the longer t_c values, or exclusion of some of the interactivation gaps at the shorter t_c values, or both.

Two channels active simultaneously

Occasionally clusters of openings contained multiple openings (i.e. more than one channel open simultaneously); such clusters were rejected. It would be useful to know how likely it is that the clusters which contained no multiple openings originated entirely from one individual channel. The method of Colquhoun & Hawkes (1990) shows that the number of consecutive single openings in the observed clusters was considerably greater than would be expected by chance if there were two identical channels present, each of which showed continuous bursting activity. However, bursting activity in each of two independent channels would not generally overlap exactly. We therefore looked for partial overlap of clusters by checking whether the gap between bursts was shorter in the middle of a cluster than at its ends; no such tendency was detectable. In order to do a more rigorous test of the hypothesis that an entire cluster originates from one individual channel, a simulation of random channel activity was done using a random number generator. The model was

$$C_2 \underset{7\cdot8}{\overset{3\cdot5}{\rightleftharpoons}} C_1 \underset{112}{\overset{265}{\rightleftharpoons}} O \underset{0\cdot01}{\overset{0\cdot47}{\rightleftharpoons}} C_3,$$

where C represents a shut state and O an open state. C_2, C_1 and O generate bursts of activity, and C_3 represents a very long-lived shut (desensitized) state, entry into which ends a cluster. The rate constants (in s^{-1}) shown mimic the records in 50 μM-GABA, with mean open time 8·9 ms, mean shut time within bursts 3·66 ms, mean number of openings per burst = 35, and mean number of bursts per cluster about 8. The mean lifetime in state C_3 was set as 100 s. Simulations of 81920 transitions in each of two independent channels, with $t_c = 50$ ms for bursts, and $t_c = 3$ s for clusters, gave 1102 bursts in 136 clusters with a mean cluster length of 6·5 s and a mean gap between clusters of 4·9 s. Of the 136 clusters, 59 came from channel 1 only, 61 came from channel 2 only, 8 contained double openings, and 8 contained openings from both channels but no double openings. Thus, of 128 clusters that contained no double openings, 94% originated entirely from one individual channel.

Other simulations were done with the mean lifetime of state C_3 set at 30 or 300 s

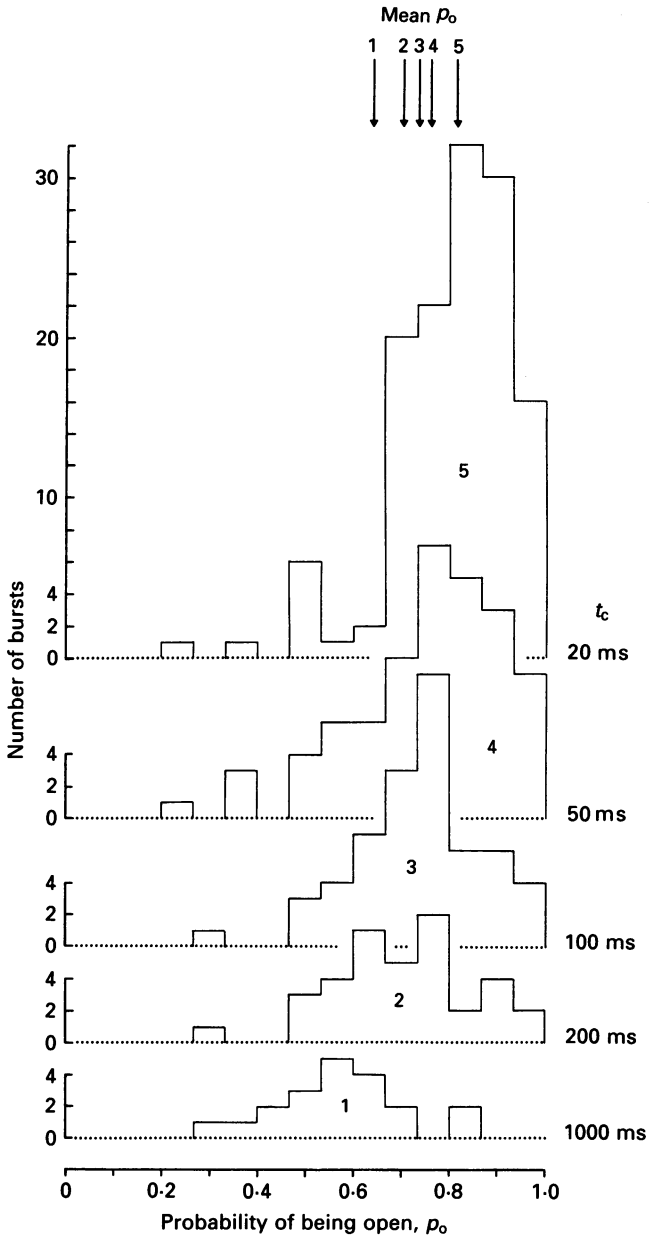


Fig. 8. Effect of selecting different values for the critical gap duration, t_c, on the spread of p_o values and on the overall mean p_o. Histograms of p_o for bursts within clusters, over a range of t_c (20–1000 ms); the t_c value used for each histogram is given on the right-hand side (from top to bottom, t_c = 20, 50, 100, 200, 1000 ms). The overall mean p_o for each t_c is indicated by the arrows (identified by their corresponding numbers) above the histograms: mean p_o = 0·64 (t_c = 1000 ms; number of bursts, n = 20), 0·71 (t_c = 200 ms; n = 37), 0·74 (t_c = 100 ms; n = 59), 0·76 (t_c = 50 ms; n = 84), and 0·81 (t_c = 20 ms, n = 131). Note the wide spread in p_o regardless of t_c, and that changing t_c from 20 to 1000 ms shifts the mean p_o by only 0·17. Results represent data from twenty clusters recorded from outside-out patches (V_m = −60 to −100 mV) exposed to 50 μM-GABA. Only bursts of more than 100 ms duration were included.

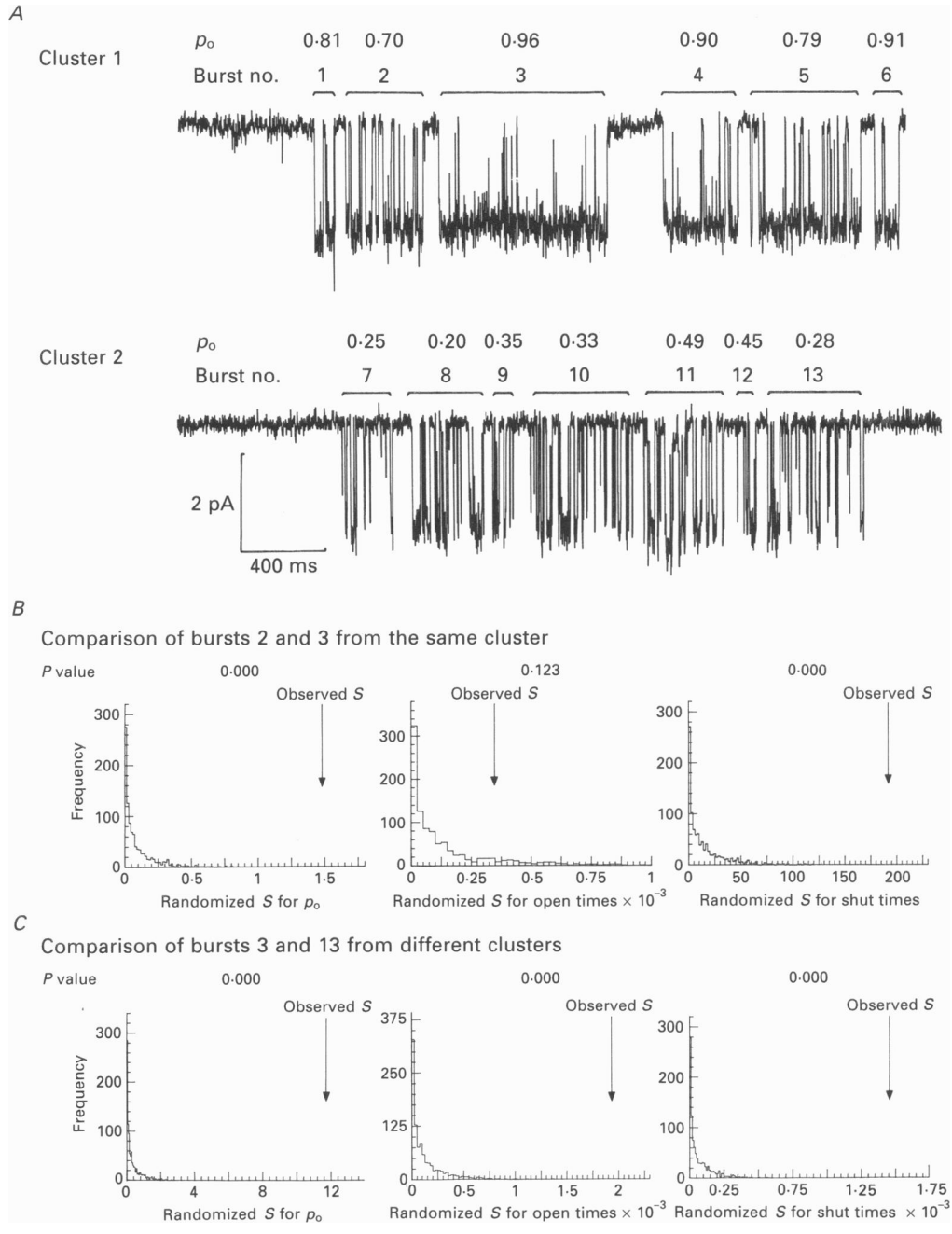


Fig. 9. Randomization test to compare bursts within a cluster and bursts from different clusters. *A*, records selected to illustrate different p_o values for bursts within a cluster and for bursts from two different clusters, in the same outside-out patch. The record shows two successive clusters. The first cluster (upper trace) occurred 2·8 s before the second cluster (lower trace; 2·8 s gap not shown). The two clusters have been subdivided into thirteen bursts (numbered burst no. 1–13 above each burst) using a t_c of 50 ms.

(rather than 100 s), and with t_c for definition of clusters set at 1 s or 10 s (rather than 3 s). The results were not greatly different: the proportion of the clusters which contained no double openings that originated from one channel only was 94–100% in each case. We may therefore conclude that most (probably over 90%) of the experimentally observed clusters will consist of openings that all come from the same individual ion channel. Changes of channel behaviour during a cluster therefore do not, in most cases, represent a difference between two channels, but rather a change in the behaviour of one channel.

Randomization test for heterogeneity of bursts

The values obtained for p_o, as well as the mean open and shut times, for bursts within the same cluster and between different clusters, were compared by means of a randomization test (see Patlak, Ortiz & Horn, 1986). The aim of this test was to examine whether the wide variability in p_o could plausibly occur if all open and closed intervals came from the same population, that is, from channels showing homogeneous behaviour.

Values of p_o, and of mean open and shut time, have been compared for sets of two to twenty bursts, for bursts of more than 200 ms duration (more than 40 openings) and at a single agonist concentration. Figure 9 shows an example of such an analysis applied to two clusters of openings. The single-channel records (Fig. 9*A*) show two clusters (cluster 1 and cluster 2) recorded from a single patch in the presence of 50 μM-GABA. These two clusters have been selected to illustrate the markedly different p_o values of bursts within the same cluster, and of bursts from different clusters. Cluster 2 occurred 2·8 s later than cluster 1. Together the two clusters were divided into thirteen bursts using a t_c of 50 ms. The p_o values for the six bursts in cluster 1 ranged from 0·70–0·96, while the p_o values of the seven bursts in the second cluster ranged from 0·20–0·49.

Figure 9*B* shows a randomization test comparison of the values of p_o, mean open time and mean shut time of bursts no. 2 and no. 3 from cluster 1 (Fig. 9*A*). The p_o values were 0·7 for burst no. 2, and 0·96 for no. 3. The histograms of randomized scatter (randomized S) were obtained under the null hypothesis that the two bursts were homogeneous, by generating 1000 pairs of randomized bursts (see Methods). There is good evidence that the true p_o and the true shut time distributions both differed between the two bursts, since the estimated proportion of randomized S values which exceeded the observed S value was zero for p_o and shut times (i.e. none of the 1000 randomized S values exceeded the observed S). However, the proportion of randomized S which exceeded the observed S for the open times was 0·123, so there

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Corresponding p_o values are also given (50 μM-GABA, $V_m = -80$ mV; temperature, 22 °C; effective filtering 670 Hz, −3 dB). *B*, histograms showing randomized scatter for p_o (left), for open times (middle) and for shut times (right) obtained under the null hypothesis that bursts no. 2 and no. 3 are homogenous (see text for details). 1000 pairs of artificial bursts were compared. The arrows indicate the level of the observed scatter for burst no. 2 and burst no. 3. The proportion of randomized S values which exceed the observed S value is indicated above each histogram (P values). *C*, histograms of randomized scatter for p_o (left), for open times (middle) for shut times (right) obtained when comparing two bursts (3 and 13) from different clusters.

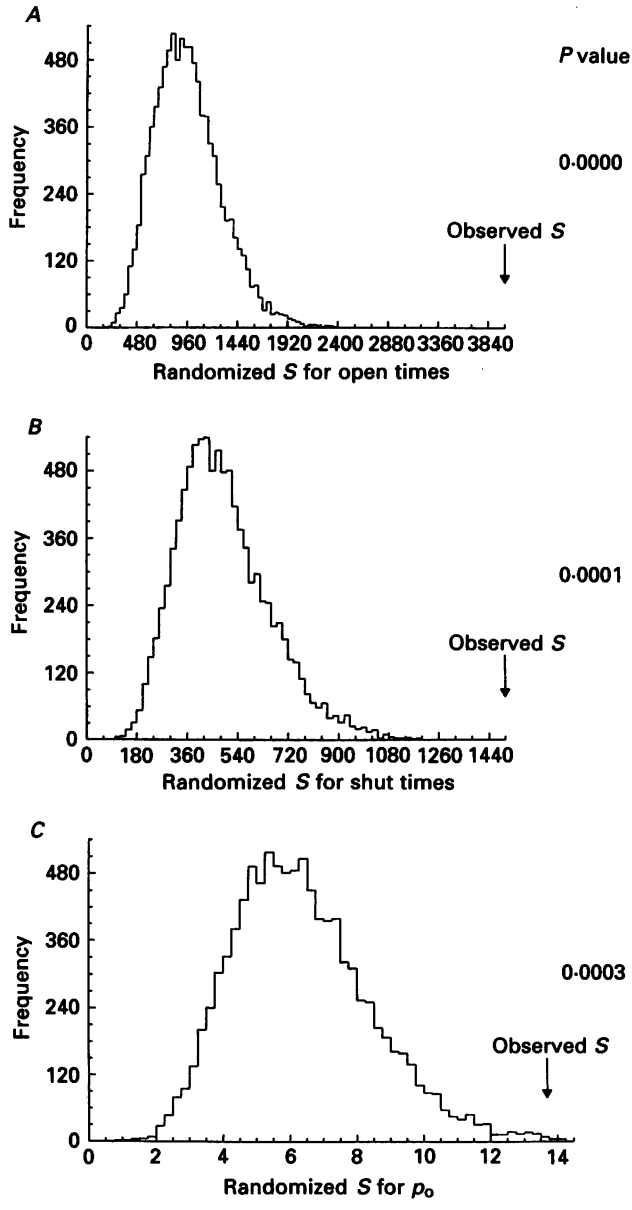

Fig. 10. Randomization test to compare twenty bursts from six clusters, recorded from one patch exposed to 50 μM-GABA; $V_m = -70$ mV; temperature, 22 °C. Bursts were defined by a t_c of 50 ms. The histograms are of randomized scatter (Randomized S) of open times (A), shut times (B) and p_o (C), obtained under the null hypothesis that the twenty bursts are homogeneous, and using 10^4 randomizations (see Methods for details). Arrows indicate the level of the observed scatter (Observed S). The proportion of randomized S values which exceeded the observed S value is shown to the right of each histogram (as P values). Note the larger scatter in open times, shut times, and p_o, for the observed bursts compared with that expected for receptor channels showing homogeneous behaviour.

was no compelling evidence that the open times differed between the two bursts. Figure 9*C* shows a similar comparison for bursts no. 3 and no. 13 (i.e. bursts from different clusters). Burst no. 3 contained sixty-four openings and $p_o = 0{\cdot}96$; burst no. 13 contained forty-one openings, $p_o = 0{\cdot}28$. Using 1000 pairs of artificial bursts (produced by randomization), the *P* values for p_o, mean open time and mean shut time, were all 0·000 indicating a difference in the kinetic properties of the two bursts.

To eliminate bias in selecting bursts, all bursts recorded from any particular outside-out patch, which satisfied the criteria previously indicated (in terms of amplitude, and of duration), were compared. Figure 10 shows the result of such a randomization test, used to compare all of the bursts that were longer than 200 ms (20 in total) recorded from a single patch exposed to 50 μM-GABA. The bursts were defined by a t_c of 50 ms and originated from six clusters. The p_o of the twenty observed bursts ranged from 0·56 to 0·89, and the grand mean p_o was 0·73. The randomization procedure was used to generate 10^4 sets of twenty artificial bursts, in order to construct histograms of randomized *S* for p_o, and for the open times and shut times. In this example the estimated *P* values were: 0·0001 for shut times, 0·0003 for p_o, and 0·0000 for open times, indicating that all three parameters were significantly different for bursts within and between the clusters, recorded in this particular patch.

In summary, the p_o, mean open time, and mean shut time have been found to differ between one channel and another (i.e. between clusters), and for the same channel at different times (i.e. between bursts within the same cluster).

Whole-cell dose–response relationship

The whole-cell dose–response relationship was also measured for comparison with the single-channel data, and with published results. The peak whole-cell responses are likely to be attenuated by desensitization though the extent of desensitization at the peak will be less than that in the single-channel records from which the 'within cluster' p_o was measured.

The whole-cell dose–response relationship illustrated in Fig. 11 contains data pooled from twelve cells (clamped at −60 mV). The normalized responses are the peak amplitudes of the current responses expressed as a percentage of the peak response to 10 μM-GABA in the same cell. This was necessary since the absolute amplitude of current responses to a given concentration of GABA, (at a particular holding potential), varied from cell to cell (up to a twofold difference). It is apparent from the relationship that at concentrations of GABA below 20 μM, the normalized responses did not vary greatly from cell to cell, in contrast to the wide variability in responses to concentrations above 20 μM. Part of this variability is undoubtedly due to differences in the extent of desensitization at the apparent peak of the whole-cell current responses.

Figure 11*B* illustrates three representative whole-cell currents recorded from the same cell in response to 5, 10 and 20 μM-GABA. There was little apparent desensitization below 10 μM-GABA, but above this concentration there was pronounced desensitization. The whole-cell dose–response relationship flattens above 30 μM-GABA, probably partly as a result of attenuation of the peak response by desensitization. By comparing Figs 6 and 11 it can be seen that the lowest concentration of GABA used to determine the p_o *vs.* GABA concentration

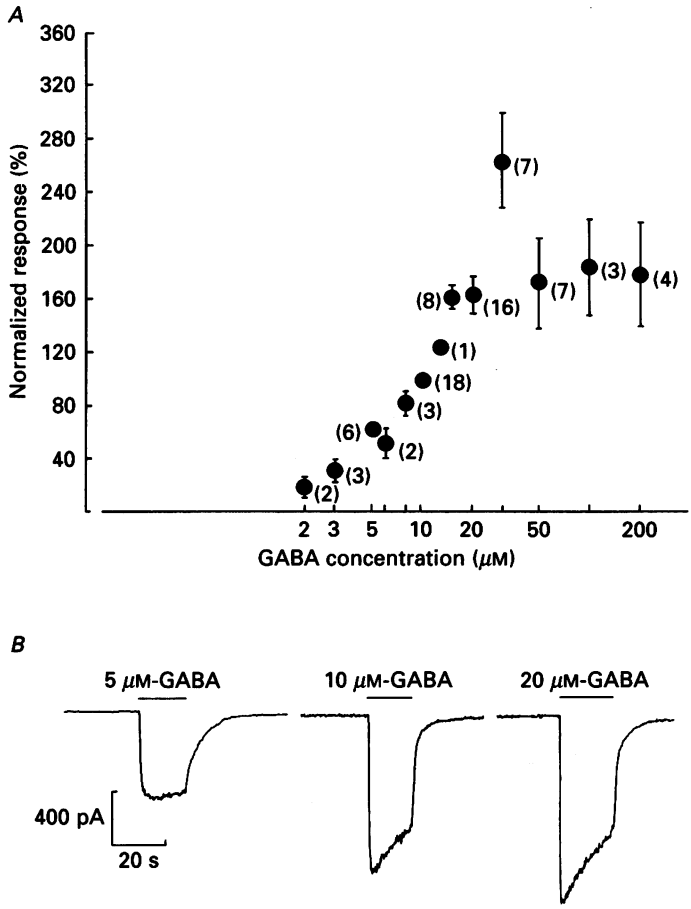

Fig. 11. *A*, whole-cell dose–response relationship for data pooled from twelve cells (V_m = −60 mV). In each cell peak responses were normalized with respect to the peak response to 10 μM-GABA. The numbers in parenthesis indicate the number of responses averaged for each point. Vertical bars represent ±S.E.M. (where these values are larger than the symbol). The concentration of GABA which produced a response of approximately 50% of the apparent maximum response, was about 10 μM; the plateau of the dose–response relationship occurred above 30 μM-GABA. *B*, representative current responses from one cell (used in *A*) evoked by 5, 10 and 20 μM-GABA. Calibration, 400 pA, 20 s.

relationship (10 μM) is close to the apparent EC_{50} of GABA in these cells. The EC_{50} of GABA was taken as the concentration which produced a response of approximately 50% of the apparent maximum response (Fig. 11*A*).

DISCUSSION

Multiple-conductance states

A conductance level of about 30 pS was the most common in the present work, and values of about 22–23, 15–18 and 7–9 pS could also be seen with reasonable reliability. However, the results are such that this cannot be regarded as an

exhaustive list. For much of the time, open channels were not in any clearly identifiable discrete conductance level, but rather appeared noisy. Some of this noisy appearance may result from the channel oscillating relatively quickly between various closely spaced and discrete conductance levels. However, the only evidence for closely spaced levels in these neurones is that (1) the open point amplitude histograms and mean low-variance amplitude histograms could, in many cases, be fitted with the sum of two or more Gaussians which had closely spaced means, and (2) when considering clusters of fairly consistent amplitude, the amplitudes clearly differed from one cluster to another in the same patch, and often by only a few picosiemens. It is interesting to note that nicotinic acetylcholine channels in rat sympathetic neurones also appear noisy in their open state (Mathie, Cull-Candy & Colquhoun, 1988). In contrast GABA receptor channels in various other types of mammalian neurones have been reported to open to a number of discrete and widely spaced multiple-conductance levels (Hamill, Bormann & Sakmann, 1983; Cottrell, Lambert & Peters, 1985; Allan & Albuquerque, 1987; Bormann *et al.* 1987; Macdonald *et al.* 1989). It is not possible, on the basis of the results presented in previous studies, to determine whether there was evidence that these GABA receptor channels also opened to closely spaced levels. Furthermore, other amino acid-activated channel types, such as mammalian central glutamate receptor channels (Cull-Candy & Usowicz, 1987; 1989; Jahr & Stevens, 1987; Cull-Candy, Howe & Ogden, 1988; Howe, Cull-Candy & Colquhoun, 1990) and glycine receptor channels (Hamill *et al.* 1983; Bormann *et al.* 1987) produce single-channel currents that are far less noisy than GABA receptor channels and also have more discrete multiple-conductance levels.

Although the conductance levels of GABA receptor channels in other cell types are reported to be more discrete than in the present work, there is little similarity in the amplitudes reported by different authors for the various conductance levels (range 6–45 pS). This may result, in part, from differences in experimental conditions, such as temperature (21–26 °C for the cited papers), pH, and chloride activities. We have found that temperature alters the single-channel conductance of GABA channels in rat sympathetic neurones (Newland, 1990). Increasing the temperature resulted in an increase in the amplitude of the main (30 pS) conductance level (Q_{10} approximately 1·3; effect of temperature on the amplitude of the other conductance levels was not investigated). Thus only modest differences would normally be expected to result from differences in these conditions.

An alternative explanation for the reported differences in the amplitudes of the conductance levels is that they reflect the presence of different forms of the GABA receptor in different cell types.

General features of the p_o curve

The maximum p_o value, 0·83, that could be observed at high GABA concentrations, was genuinely less than 1·0. It is unlikely that this resulted from rapid open channel block by GABA molecules, since the apparent conductance of the single channels was not reduced in the presence of high concentrations of GABA. One possible explanation for the low maximum p_o is that short desensitization gaps were inadvertently included in the bursts. As discussed in the Results, ambiguities arose

in trying to decide whether the briefer shut periods within clusters (of less than 100 ms) were spent in short-lived desensitized states or whether they were spent in the resting (i.e. activatable) state of the non-desensitized receptor. We feel a more likely explanation for the low maximum p_o is that GABA may be a partial agonist. That is, the opening rate of the agonist bound receptor channel may be little greater than the shutting rate into the bound but activatable state, (i.e. low β/α; see Colquhoun & Ogden, 1988); a maximum p_o of 0·83 corresponds with $\beta/\alpha = 4{\cdot}9$.

Low values of β/α (between 2·8 and 6·6) have previously been inferred from observations of the activation of GABA receptors by low concentrations of GABA, in bovine chromaffin cells and mouse spinal neurones (Sakmann, Hamill & Bormann, 1983; Bormann & Clapham, 1985). In these cases a simple sequential reaction scheme with one open state was considered adequate to describe the gating of GABA channels. Based on this scheme, values of β and α were derived according to Colquhoun & Hawkes (1977, 1982). Recently, however, comprehensive analysis of GABA single-channel currents in cultured chick cerebral and mouse spinal neurones, has led Macdonald *et al.* (1989) and Weiss & Magleby (1989) to propose that the kinetics of the main conductance state is far more complex than can be described by a simple sequential scheme. It is therefore difficult, at present, to give values for β/α.

At high concentrations of GABA we found that the gaps between clusters usually greatly exceeded the duration of a cluster, even when there were several channels in the patch. It is therefore expected that one of the macroscopic desensitization time constants will be comparable to the mean cluster length, which in the present study was about 3·8 s at 50 μM-GABA. This is similar to the most rapid time constant of macroscopic desensitization reported for frog spinal neurones (Akaike *et al.* 1986) and for *Aplysia* neurones (Ikemoto *et al.* 1988).

Furthermore, the GABA concentration *vs.* whole-cell peak current relationship described here did not have a clearly defined maximum, probably as a result of attenuation of the peak current response by fast receptor desensitization. It was therefore not reasonable to estimate precisely the EC_{50} for GABA or the Hill coefficient for GABA from this whole-cell dose–response relationship. However, a GABA concentration of about 10 μM produced a response that was approximately 50% of the apparent maximum response amplitude (i.e. the EC_{50} was approximately 10 μM). This value is in reasonable agreement with EC_{50} values determined for various other isolated cell preparations in which the concentration of GABA was known (i.e. where there were no problems with GABA uptake, metabolism or diffusion). EC_{50} values of between 10 and 42 μM have been reported for bullfrog dorsal root ganglion cells (Akaike, Hattori, Oomura & Carpenter, 1985), chick spinal neurones (Choi & Fischbach, 1981) and *Xenopus* oocytes injected with the mRNA coding for bovine $GABA_A$ receptors (e.g. Levitan *et al.* 1988). It is probable that fast desensitization of GABA receptors also distorted the apparent dose–response relationships determined in some, if not all of these cases, unlike the situation in isolated patches where desensitization gaps can, in principle, be excluded.

Heterogeneous properties of GABA receptor channels

The probability of being open, as well as the mean open times and mean shut times were all found to differ not only between one channel and another (i.e between clusters), but probably for the same channel at different times (i.e between bursts

within a cluster). These differences are far greater than those expected for a channel which behaves homogeneously. Our finding of kinetic differences between bursts within a single cluster strongly suggests that there is at least one population of GABA receptors whose activity changes relatively slowly with time.

Low concentrations of GABA have been used to examine the kinetics of GABA receptor channels in various vertebrate cell types, including various mammalian neurones (Sakmann, Bormann & Hamill, 1983; Bormann & Clapham, 1985; Mathers & Wang, 1988; Macdonald *et al.* 1989) and chick neurones (Weiss & Magleby, 1989). At concentrations of GABA below 5 μM the main conductance state of the GABA receptor channels in these preparations appears to behave homogeneously. However, in none of these studies were responses to high agonist concentrations recorded. So it is not known whether the GABA channels in other neurones show the sort of heterogeneity of open and shut times reported here.

A channel which can switch between different kinetic 'modes' is not unprecedented. The term 'mode' is used here merely to refer to a subset of open and closed states of the receptor channel within which the channel may oscillate for relatively long periods of time, before switching to a different subset ('mode') in which open times and/or closed times are different. Rare, but sudden, changes in the kinetics of an individual channel have been observed for other agonist-activated channels, including glutamate receptors in locust muscles (Patlak, Gration & Usherwood, 1979; Cull-Candy *et al.* 1981) and nicotinic acetylcholine receptors in *Xenopus* myocytes (Auerbach & Lingle, 1986). Voltage-activated ion channels also occasionally switch between different kinetic states, as observed for sodium channels in adult frog skeletal muscle (Patlak *et al.* 1986) and calcium channels in cardiac cells (Hess, Lansman & Tsien, 1984). Similarly, Blatz & Magleby (1986) have reported the existence of distinct kinetic modes of spontaneous 'fast' chloride channels in cultured rat skeletal muscle, as have McManus & Magleby (1988) for large-conductance calcium-activated potassium channels in the same preparation. Nothing is known of the mechanism underlying switching in any of these cases. The present work demonstrates this sort of behaviour in single GABA channels in a mammalian neurone.

The presence of two (or more) kinetically distinct populations of GABA receptors, rather than one type with modal behaviour, has been suggested previously. Cash & Subbarao (1987) detected two phases of desensitization of GABA receptors, each with first-order kinetics, when measured by the transmembrane flux of $^{36}Cl^-$ in sealed rat brain membrane vesicles, using quenched-flow techniques. The two phases were thought to originate from two separate populations of GABA receptors since, at a concentration of GABA below saturation level, the relative Cl^- flux activities during the two phases were concentration independent. Similarly, Akaike *et al.* (1986) observed that the rate of activation of whole-cell GABA currents in frog sensory neurones was composed of two exponential components which had different concentration dependencies. The finding that these two components recovered from desensitization with different time courses, was taken as evidence for at least two receptor subtypes. However, such observations could equally arise from a single receptor type with complex desensitization behaviour, as has been observed for endplate nicotinic receptors (Feltz & Trautmann, 1982; Cachelin & Colquhoun, 1989). Yashui, Ishizuka & Akaike (1985) had previously suggested the presence of

kinetically distinct populations of $GABA_A$ receptors in frog sensory neurones, based on results from the separate analysis of GABA current noise during the peak and the steady-state phases of whole-cell responses in these neurones. The non-linearity of a plot of $\sigma^2/\bar{I}$ *vs.* $\bar{I}$, where $\bar{I}$ is the whole-cell current amplitude and σ^2 is the current variance, was taken to indicate heterogeneity of the GABA receptor, the different subtypes being present in different numbers and having different conductances (approximately 4, 13 and 25 pS). However, it is well documented that multiple-conductance levels often originate from the same $GABA_A$ receptor channel, rather than originating from different channel types, as observed in mammalian neurones (e.g. Bormann *et al.* 1987). There is also much indirect evidence, from reversible binding studies with benzodiazepine ligands and from [^{3}H]flunitrazepam photo-affinity labelling, that there are at least two subtypes of 'central' mammalian $GABA_A$/benzodiazepine receptors (reviewed by Squires, 1983; Sieghart, 1985, 1989).

Studies using molecular cloning techniques have recently provided direct evidence for the existence, in mammalian and chick brain, of several isoforms of the α- (Levitan *et al.* 1988) and β-subunit (Yemer, Schofield, Draguhn, Werner, Köhler & Seeburg, 1989) which constitute part of the GABA receptor channel. Studies using SDS–polyacrylamide gel electrophoresis have also shown the existence of several forms of the α- and β-subunits (identified by binding of either [^{3}H]flunitrazepam or [^{3}H]muscimol and either α- or β-subunit-specific antibodies) (Fuchs, Möhler & Sieghart, 1988; Fuchs & Sieghart, 1989). Furthermore, there is different regional expression of the various α-isoforms (Levitan *et al.* 1988; Wisden, Morris, Darlison, Hunt & Barnhard, 1988). Additional subunits of the $GABA_A$ receptor have also recently been identified, namely a γ-subunit (Pritchett, Sontheimer, Shivers, Ymer, Kettenmann, Schofield & Seeburg, 1989) and a σ-subunit (Shivers, Killisch, Sprengel, Sontheimer, Köhler, Schofield & Seeburg, 1989). Two recent studies indicate that these different subunits may form functionally different $GABA_A$ receptors. Thus Levitan *et al.* (1988) demonstrated that expression of three different α-subunit isoforms with a single β-subunit, in *Xenopus* oocytes, formed functional receptors (lacking benzodiazepine sensitivity) for which GABA had apparently different potencies. Pritchett *et al.* (1989) observed that expression of these three α-subunits in combination with a β_1- and a γ_2-subunit in mammalian cells, formed receptors for which several benzodiazepine ligands had variable binding affinities, similar to type I and type II benzodiazepine receptors. Taken together, the results from binding studies and molecular cloning suggest the presence of several structurally, and possibly functionally, different $GABA_A$ receptor channels in mammalian brain.

The present work provides strong evidence for at least one structurally homogeneous population of $GABA_A$ receptor channels whose activity changes with time. It is also quite possible that there are two or more distinct populations of $GABA_A$ receptor channels which exhibit different functional characteristics.

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