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γ-Aminobutyric acid- and piperazine-activated single-channel currents from *Ascaris suum* body muscle

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1 γ-Aminobutyric acid (GABA)- and piperazine-activated single-channel currents were recorded from the bag region of the somatic muscle of the nematode parasite *Ascaris suum*. Cell-attached and outside-out patch-clamp techniques were used. Clean membranes were routinely prepared using collagenase.

2 GABA (concentrations greater than 1 μM) or piperazine (concentrations greater than 200 μM) applied to the extracellular surface of the patches brought about the opening of channels producing rectangular shaped current pulses of varying duration but essentially constant amplitude. The I/V relationships of the single-channel currents for both agonists were linear and had conductances in the region of 22 pS (in symmetrical 170 mM Cl^-). The reversal potential was near 0 mV when Cl^- was equally distributed on both sides of the membrane. Occasionally two subconductance states were seen. The mainstate single-channel permeability was estimated to be 4×10^{-14} $cm^3 s^{-1}$.

3 At low concentrations of GABA (3–4 μM), the effective mean channel open time was in the region of 32 ms (−75 mV, 22°C, cell-attached patches). At low concentrations of piperazine (500 μM) the effective mean open channel lifetime was shorter, in the region of 14 ms (−75 mV, 22°C cell-attached patches). For each agonist the channel open lifetime distributions were best described by the sum of two exponentials suggesting two open mainstates. Channel openings occurred as single events and in bursts with brief closed periods within bursts. The channel closed time histograms at these concentrations were best described by the sum of up to three exponentials, suggesting the presence of three closed states. Channel open times showed no appreciable voltage sensitivity.

4 Before desensitization, increases in agonist concentration produced an increase in the probability of the channel being open. The increased probability was associated with an increase in the frequency of channel opening, an increase in the effective mean channel open time, an increase in burst duration, an increase in the number of openings per burst, together with a reduction in the proportion of brief openings.

5 Desensitization was seen as a decline in the probability of the channel being opened during prolonged applications of agonist. It was associated with the appearance of very long (seconds) closed periods. The distributions of the closed channel times were then best described by up to four exponentials.

Introduction

Nematode parasites continue to inflict much suffering on human and animal hosts. Approximately one in five of the world's human population is infected with intestinal nematodes (Standen, 1975), while infestations in domestic animals are a major source of economic loss. Anthelmintics make a substantial contribution to the control of these parasites.

The electrophysiological effects of anthelmintics on nematodes can be studied in the parasite *Ascaris suum*. This is because this pig parasite has large muscle cells which have allowed easy intracellular recording (e.g. Jarman, 1959). The muscle cells of *Ascaris suum* are composed of four parts: the bag, spindle, arm and syncytium, (Figure 1). The bag contains the nucleus; the spindle is the contractile portion; the arm conducts action potentials from the neuromuscular junction at the syncytium (De Bell *et al.*, 1963).

It has been shown that the important anthelmintic, piperazine, acts as a low potency agonist at extrasynaptic γ-aminobutyric acid (GABA) receptors on

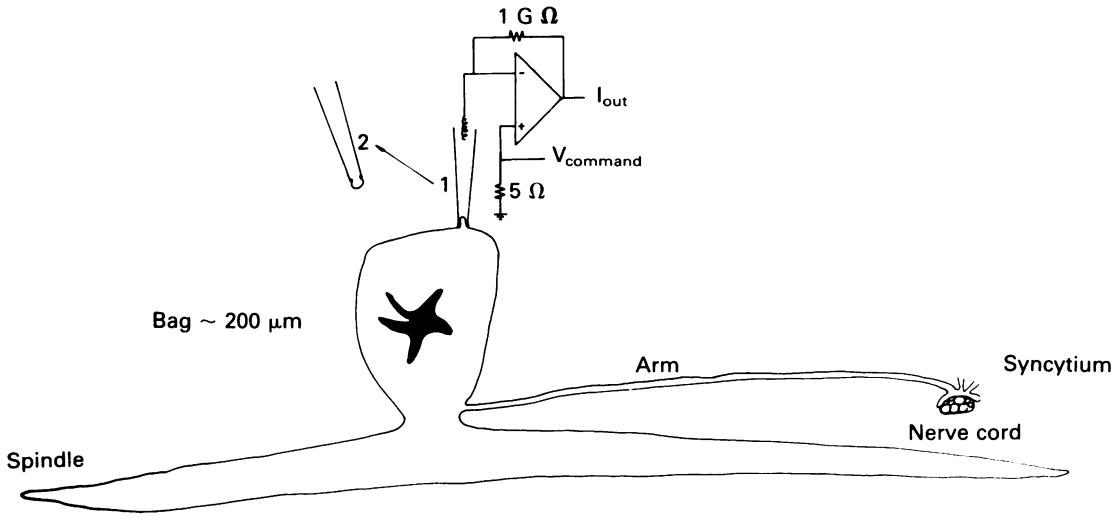

Figure 1 Diagram of *Ascaris* muscle cell and recording technique. An electrode recording from a cell-attached patch (1) is seen applied to the bag region of the muscle cell. An outside-out patch (2) is also shown. The transpatch current was monitored at 'I out'. The potential of the patch electrode was controlled at 'V command'.

the bag region of the muscle and that activation of the receptors gives rise to an increase in Cl^- conductance (Martin, 1982). As a further study of effects of agonists on these extra-synaptic receptor operated channels it was decided to carry out a patch-clamp study in the manner of Hamill *et al.* (1981). This paper describes observations which allow a description of the unitary conductance and the kinetic behaviour of single extra-synaptic receptor operated channels opened by GABA and piperazine. An initial account has already appeared (Martin 1983).

Methods

In general the methods of Hamill *et al.* (1981) were followed but the application to *Ascaris* is described below.

The preparation

Specimens of *Ascaris suum* were collected from the local slaughter house and maintained in Locke solution (replaced daily) at 32°C in a water bath. The *Ascaris* were used for experiments within four days. A 2 cm flap preparation made from the anterior region of the worm was pinned on to Sylgard in an experimental chamber which was surrounded by a water jacket to maintain the temperature at 22–25°C. Generally the smaller parasites (about 10 cm) were used rather than the larger ones.

Enzyme treatment

The preparations were routinely treated with collagenase (1 mg ml^{-1}) for 1 h to remove the superficial connective tissue and extracellular matrix which normally cover the bag membrane. Electron microscopy confirmed that this enzyme treatment was sufficient to prepare 'clean' membranes. The addition of this enzyme at 22–25°C to the preparation was associated with a decline in the bag resting membrane potential from about −35 mV to less than −7 mV. There was also a small increase in the bag input conductance. Despite this, it was shown using intracellular recording techniques that bath application of GABA still produced large reversible increases in bag input conductance as in the control period before enzyme treatment. However, as a result GABA produced little or no hyperpolarization of the bag membrane. Estimation of the GABA reversal potential using current pulse injections (Martin, 1980) showed that this had declined from −65 mV to between −7 mV and 0 mV. This observation indicated an increase in the intracellular Cl^- concentration to near extracellular levels. During patch experiments membrane potentials were checked using the whole-cell recording mode. However, it was not possible to clamp the cell effectively in this way because of the high input conductance of the cell (2–3 μS).

The effect of collagenase on membrane potential has not been described in other muscle preparations under similar circumstances. As a further examination

of the action of collagenase, the time and temperature dependency of the effect on potential was examined using intracellular recording electrodes. It was found at 22–25°C that the membrane potential declined gradually to less than −7 mV within 20 min of applying the collagenase. The membrane potential did not recover at 22–25°C during further periods of examination (up to 6 h). When the temperature of the preparation was raised to 32–37°C it was found that there was a partial recovery of the resting membrane potential within 30 min. These observations could be explained by the increase in the effective surface area of the membrane exposed to Ringer solution as a result of collagenase action together with the suppression at 22–25°C of an active transport mechanism which is implicated in maintaining the resting membrane potential (Brading & Caldwell, 1971). It was not advantageous to record from preparations at higher temperatures since spontaneous depolarizing potentials and movement can be present (Martin, 1980); further details of the flap preparation are available (Martin, 1980).

Ringer solution

The preparation was maintained in a low-Ca^{2+} Ringer solution containing (mM): NaCl 135, KCl 3.0, $MgCl_2$ 15.7, glucose 3.0 and Tris 5.0 adjusted to pH 7.6 with maleic acid. A low-Ca^{2+} Ringer was used for two reasons: firstly, to reduce spontaneous muscle contraction; secondly, to reduce spontaneous Ca^{2+} activated ion channels. Initial experiments showed that spontaneous high conductance (> 100 pS) channels were frequently present in membrane patches taken from the bag. These channels were abolished when the preparation was perfused with low- Ca^{2+} Ringer solution.

Patch electrode solution

The patch electrode was filled with a solution containing (mM): CsCl 140, $MgCl_2$ 15.7 and Tris 5.0 adjusted to pH 7.6 with maleic acid. The Cs^+-rich solution was used to suppress any K^+ currents. EGTA was not included as a Ca^{2+} buffer because its chemical structure suggested the possibility of an interaction with the GABA receptors: this was not examined systematically.

Recording

Patch electrodes were made from Hawksley microhaematocrit capillary tubes (cat. no. 1604) and had a resistance of 1 MΩ with an outside tip diameter of 1 μm. They were connected by a Clarke's electromedical suction pipette holder to the input of the current-voltage converter. The current-voltage converter was laboratory made and based on a Teledyne Philbrick 1035 op-amp and a 1 GΩ (Welwyn) feedback resistor. Recordings were amplified and filtered at 1 kHz (−3 dB). The patch electrodes were coated to near the tip with Sylgard to improve the frequency response of the recording system.

A cell-attached patch was formed when a patch electrode was pushed gently against the bag membrane and suction was applied to the pipette. After suction the seal resistance between the pipette tip and membrane patch gradually increased to greater than 1 GΩ. It was then possible to alter the transpatch potential by altering the patch electrode potential. Outside-out patches were prepared by rupturing the cell- attached patch by applying a high transpatch potential (> 150 mV) and then gently withdrawing the patch electrode from the bag. Under favourable circumstances the patch resealed in the outside-out conformation (Hamill *et al.*, 1981).

Data processing

Responses were monitored on an oscilloscope and recorded on a Racal Thermionic Store Four FM tape recorder (slowest frequency response 1.25 kHz, −1 dB). Initially single-channel current responses were played back from the tape at one quarter the original speed on to a Devices MX212 two channel pen recorder. The channel open and closed times were then analysed by hand with the aid of a Reichert Jung videoplan. Subsequently recordings on tape were analysed with the aid of a Cromenco microcomputer programmed to measure channel open and closed times. The data were played back from the tape, sampled and digitized every 200 or 400 μs and continually monitored and adjusted for slow d.c. drifts. An increase in current of greater than about 70% of the unit channel current was recorded as a channel opening. After an opening, a decrease in the current to less than about 50% of the single channel current was recorded as channel closing. The programme then determined and stored the durations of each open and closed interval in order of their occurrence (as the integer number of sample intervals). For each patch record mean values, frequency histograms and their semi-log plots of open and closed times were displayed and plotted by the microcomputer programme. The numbers representing open and closed times were transferred over the local area network to a mainframe computer for further analysis. Effective mean open times are here quoted as the observed values without correction for unresolved short intervals. Analysis of the data by hand or computer gave essentially the same estimates for effective mean open and closed times. However, these values were affected by the threshold settings since measurement of open times is affected by the number of rapid brief closings or 'flickerings' that

are detected by this analysis. Measurements of open times in the presence of GABA or piperazine were therefore made using very similar threshold settings for both sets of data.

Statistics

It was assumed that the channel open or closed times were distributed as a mixture of m exponentials. The probability density function for the open or closed time of T ms is then

$$f(T) = \sum_{i=1}^{m} \frac{K_i}{U_i} e^{-T/Ui} \qquad -(1)$$

Where the ith exponential has a mean U (>0) and is present in the proportion K_i ($1 \geq K_i \geq 0$) and

$$\sum_{i=1}^{m} K_i = 1$$

When sampling frequency is δ ms the probability that an open or closed interval of T ms duration is recorded as an opening or closing of j intervals (where j is an integer) is

$$\begin{aligned} p(j|T) &= [T/\delta] + 1 - T/\delta && \text{if } j = [T/\delta] \\ &= T/\delta - [T/\delta] && \text{if } j = [T/\delta] + 1 \\ &= 0 && \text{otherwise} \end{aligned} \qquad -(2)$$

where [z] denotes 'the integer part of z'.

Combining (1) and (2) the probability that an opening or closing is recorded as being between j and k intervals (inclusively) is

$$P(j,k) = \int_0^{\infty} \sum_{l=j}^{k} p(l|T)\, f(T)\, dT$$

$$= \sum_{i=1}^{m} K_i\, U_i/\delta\, (e^{-\delta j/Ui} - e^{-\delta(k+1)/Uj})\, (e^{-\delta/Ui} - 1) \qquad -(3)$$

If open or closed times less than T_0 ms or greater than T_1 ms are (partially) censored that it is not observed, then (3) holds between the limits j_0 and k_0 where

$$j_0 = |\, T_0/\delta - \textstyle\sum\, | + 2$$

and

$$k_0 = |\, T_1/\delta + \textstyle\sum\, | - 2$$

and $\sum$ is an arbitrarily small positive number.

Conditional on the observed intervals being between j_0 and k_0

$$P(j,k) = \frac{\sum_{i=1}^{m} K_i\, U_i/\delta\, (e^{-\delta j/Ui} - e^{-\delta(k+1)/Ui})\, (e^{-\delta/Ui} - 1)}{\sum_{i=1}^{m} K_i\, U_i/\delta\, (e^{-\delta j0/Ui} - e^{-\delta(k0+1)/Ui})\, (e^{-\delta/Ui} - 1)} \qquad -(4)$$

which has the property that

$P(j_0, k_0) = 1$.

Examination of the open and closed time histograms show that openings less than 1 ms were not well detected and measured. Therefore, during the analysis T_0 was taken as 1 ms (Colquhoun & Sigworth 1983). When δ equals 0.2 ms j_0 was 6 and when δ equals 0.4 ms j_0 was 4. The selection of periods of observation inevitably led to censoring of long open or closed times so k_0 was set to 30,000.

If the observations are independent then the negative log-likelihood is

$$L = -\sum_{l=1}^{n} l_n P(Y_l, Y_l)$$

where P is given in (4), and Y_l is a sample of open or closed times. Parameters K and U in P were estimated from Y by minimizing L. This was carried out numerically by iterative refinement from initial guesses at the parameter values. Parameters were estimated for m = 1, 2, 3 for open times and m = 1, 2, 3, 4, 5 for closed times. It was taken that a decrease in the negative log-likelihoods of 3.0 or more for each addition of one extra exponential component indicated a significant improvement in fit.

The parameter estimates for best fitting models were then determined together with approximate standard errors based on a quadratic approximation to L. The goodness of fit of the best fit models was tested by calculating the chi-squared statistic and found to be adequate. It is pointed out that the fitting of exponentials in this way ignores time sequencing in the data which ought to carry important information when fitting compartmental models.

Drugs

The drugs used were GABA and piperazine hexahydrate. The enzyme used was collagenase (C-0130). All drugs were obtained from Sigma.

Results

The following experiments were carried out on muscle cells from 45 preparations. GABA- or piperazine-activated channels were recorded from 45 cell-attached patches and 15 outside-out patches. GABA- or

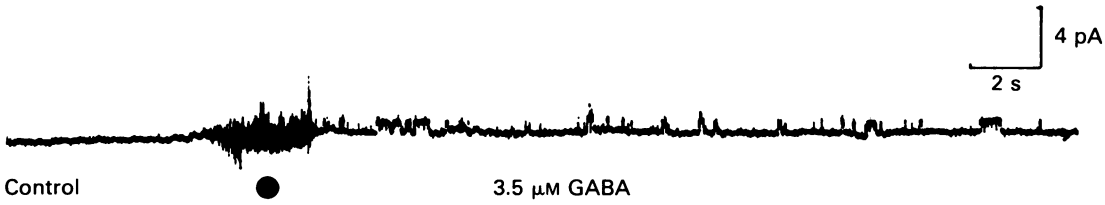

Figure 2 Effect of GABA bath-application on an outside-out patch: GABA 3.5 μM was bath-applied during the artefact marked (●). It produced rectangular outward (upward) currents of about 1 pA amplitude but of varying duration (mean open time 32 ms). Up to two channels were present in this patch. After a period of about 5 min the channel activity became quiet presumably due to desensitization (not shown). Transpatch potential + 40 mV, 170 mM Cl^- on both sides of the membrane. Although GABA 3.5 μM was also present in the patch electrode, no channels were observed prior to bath-application.

piperazine-induced channels were recorded in about 15% of cell-attached patches forming giga-seals. Single-channels with similar conductances and open times to GABA or piperazine channels were not recorded when the agonists were omitted from the patch electrode in 56 consecutive cell-attached patches, although other spontaneous ion channels were very occasionally seen. The formation of outside-out patches was generally more difficult; only 8% of cell-attached patches with giga-seals led to the production of successful outside-out patches. Observations on GABA and piperazine channel open times and conductances from the two patch types were not detectably different.

Channel activation

Figure 2 shows the typical effect of bath-application of 3.5 μM GABA to an outside-out patch with a transmembrane potential of + 40 mV. Following application of the agonist there appeared up to two rectangular outward current pulses which lasted for varying durations. The amplitude of the channel currents was essentially constant at the fixed transmembrane potential.

In the experiment illustrated in Figure 2, GABA (3.5 μM) was also present in the patch electrode (on the intracellular side of the outside- out patch) but no channel currents were observed prior to the external

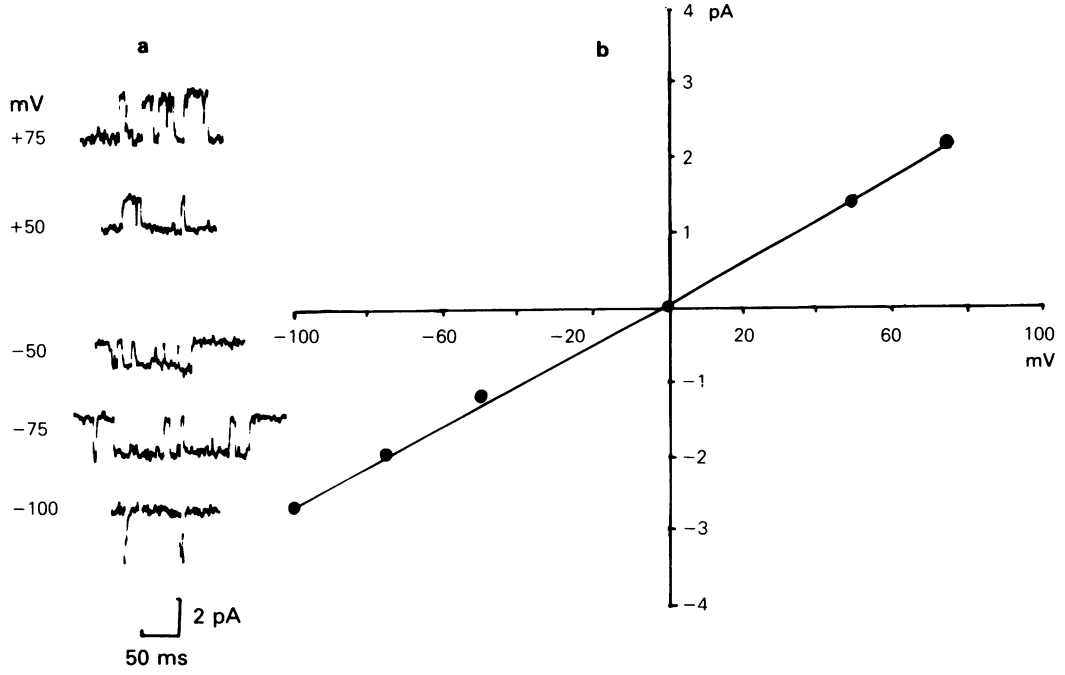

Figure 3 Current-voltage relationship of a GABA single channel. Single channel currents produced by GABA 7.5 μM using an isolated outside-out patch recording are seen at different transpatch potentials in (a). The current-voltage relationship (linear slope G = 26 pS) is plotted in (b): ordinate scale, single-channel current amplitude; abscissa scale, transpatch potential. The reversal potential was near 0 mV.

application of the agonist, so this observation is consistent with the receptor binding site only being present on the external surface of the membrane.

Channel conductance

In order to determine the channel conductance the amplitudes of the current steps at different transpatch potentials were measured. The conductance was then determined from the slope of the current voltage relationship. Figure 3 shows a typical (linear) current voltage relationship obtained between + 75 and − 100 mV from an isolated outside-out patch with GABA as the agonist. The reversal potential with Cs^+ as the main cation in the patch electrode and symmetrical Cl^- across the patch was near 0 mV. Reversal potentials close to 0 mV were also observed with cell-attached patch recordings (not shown) and enzyme-treated preparations (see Methods) where the intracellular Cl^- concentrations were found to approach extracellular concentrations. These observations on the reversal potential are consistent with the channel currents being carried by Cl^- ions. The slope of the current-voltage relationship shown in Figure 3 gave a value of 26 pS for the channel conductance. The mean estimates for the channel conductance using isolated-patches were GABA 22.0 ± 1.2 pS (mean ± s.e., $n = 11$) and in two piperazine experiments 19.0 pS: the mean estimates from cell-attached patches were GABA 21.7 ± 1.2 pS (mean ± s.e., $n = 11$) and piperazine 21.4 ± 1.5 pS (mean ± s.e., $n = 11$). There was no significant difference (t test) between any of these values. It was concluded from these observations that the channels opened by GABA and piperazine have the same mainstate conductance.

Subconductance states

Occasionally with GABA or piperazine the amplitudes of the single channel currents did not reach their full level during opening or closing but found levels approximately 30% or 70% of the full levels, (Figure 4). This was interpreted as indicating the presence of two subconductance states. However, openings of reduced amplitude comprised only a small fraction of the total open time. For example, in one experiment with 20 μM GABA as the agonist (− 75 mV cell-attached patch) less than 2% of the total open time was in any of the subconductance states. It was unlikely that the reduced conductance states arose from simultaneous opening of separate channels of opposite polarity since they were never observed at these potentials in isolation. Hamill *et al.* (1983) have also described multiple conductance states in cultured mouse spinal cord neurones for GABA and glycine channels.

Single channel permeability

Although channel conductances of around 22 pS were observed this quantity does not take account of the Cl^- concentration on each side of the membrane; channel conductances with more physiological Cl^- concentrations present are expected to be lower (Sakmann *et al.*, 1983). However, if constant field conditions were assumed, it was possible to estimate the single channel permeability. For example, a channel current of 1.57 pA was observed to flow with a driving force of − 70 mV (G = 22 pS) across the patch. An equal Cl^- distribution of 170 mM gives rise to an estimate of around 4×10^{-14} $cm^3 s^{-1}$ for the mainstate

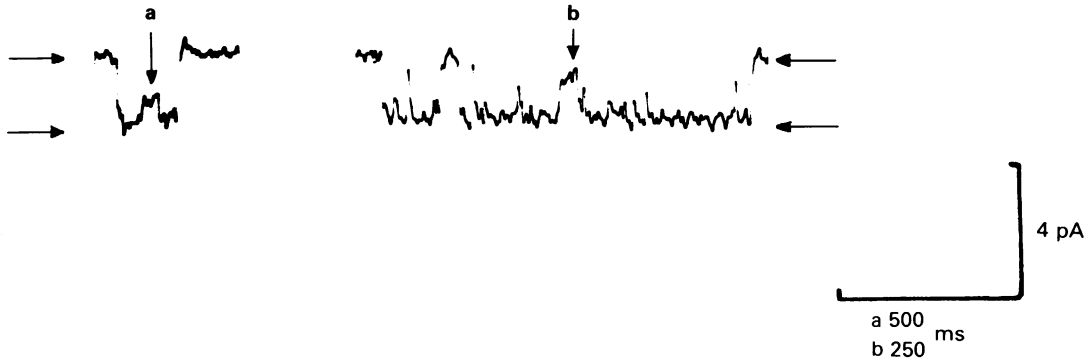

Figure 4 Subconductance states. Two cell-attached patch recordings (inward currents downwards, transpatch potential − 75 mV) are shown. (a) GABA 3 μM as agonist shows a subconductance state marked with the vertical arrow of about 70% of the mainstate. (b) Piperazine 1 mM as agonist shows a subconductance state marked with a vertical arrow which is about 30% of the mainstate. Horizontal arrows mark the main open state amplitude.

single channel permeability. The current of 1.57 pA corresponds to the movement of 10 million Cl^- ions every second through the ion channel. If the Cl^- ions pass in single file separated by a minimum distance of twice the Pauling radii (3.9 Å) a mean velocity through the membrane of around 4 mm s^{-1} would be required. Bormann *et al.* (1983) have estimated the permeability of GABA activated channels in cultured rat hippocampal neurones to be 6.4×10^{-14} cm^3 s^{-1}.

The duration of channel open times

Figure 5a shows a typical example of a cell-attached patch recording with 3 μM GABA as the agonist; the transpatch potential was − 75 mV. In this experiment the effective mean open time was found to be 34 ms. The distribution of channel open times for the experiment illustrated in Figure 5 is shown in the histogram of Figure 6a. The overall mean estimate from other experiments under the same conditions of concentration and potential for the GABA open times was 30.5 ± 2.7 ms (mean ± s.e., $n = 6$). When 500 μM piperazine was used as the agonist instead of GABA, shorter mean open channel times were observed. Figure 5b shows a typical recording using a cell-attached patch at − 75 mV. The effective mean channel open time in this experiment was 18 ms. Figure 7a shows the distribution of the piperazine open channel times observed for the experiment illustrated in Figure 5b. The mean estimate for the piperazine channel open times obtained from experiments under the same conditions of concentration and potential was

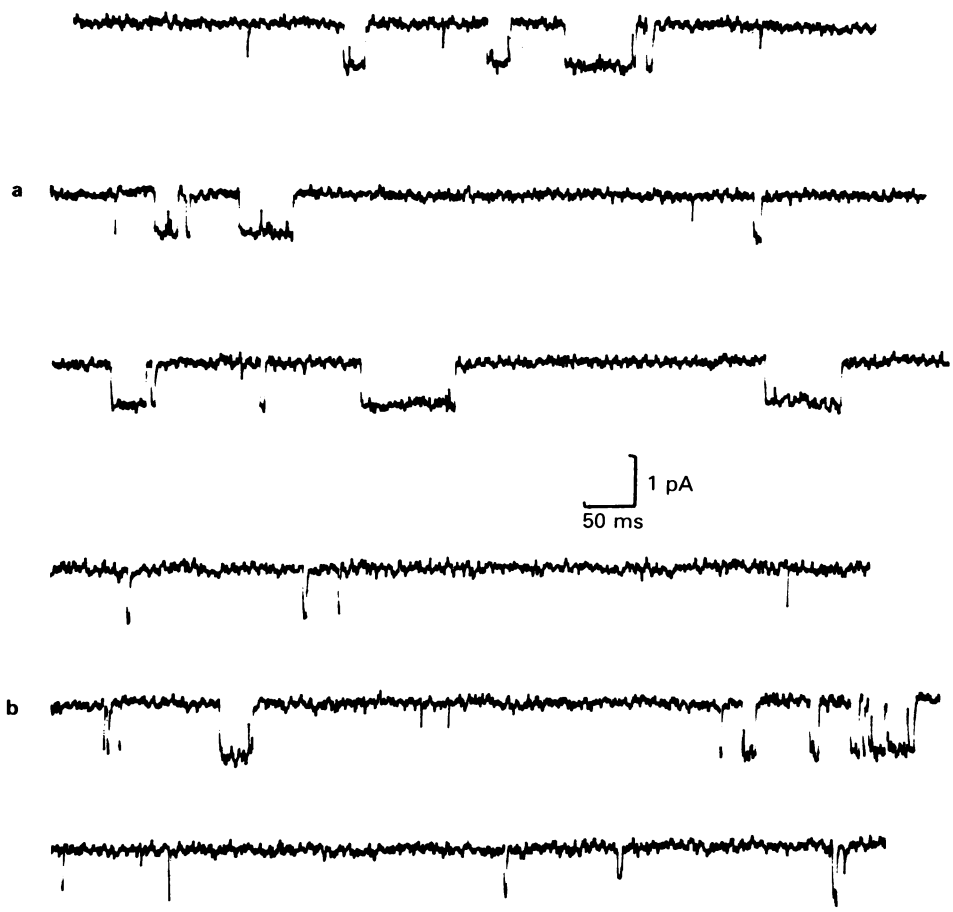

Figure 5 Continuous single-channel recordings using low agonist concentrations, cell-attached patches, transpatch potential − 75 mV, Cl^- concentration equal on both sides of membrane. Brief and longer openings are seen (downwards, inward current). Some brief closings giving rise to bursts are seen: (a) three traces GABA 3 μM as agonist; (b) three traces piperazine 500 μM as agonist. Effective channel open times: GABA, 33 ms; piperazine, 18 ms. Measured burst durations: GABA 39 ms; piperazine 24 ms.

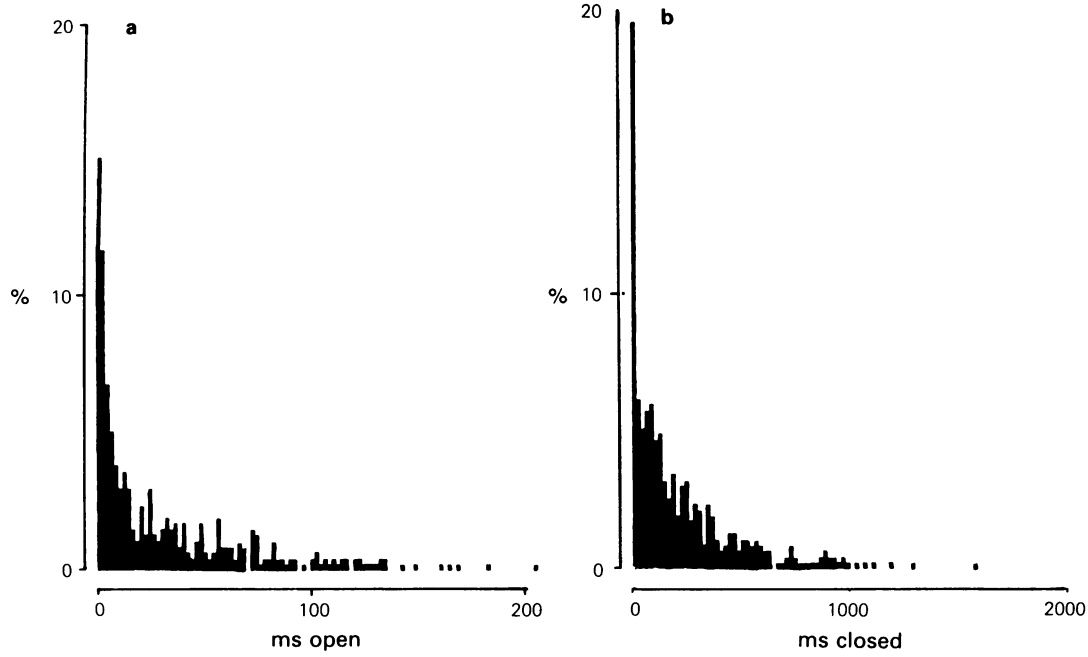

Figure 6 Distributions of GABA open and closed times. A cell-attached patch with GABA 3 μM as agonist and transpatch potential of − 75 mV was used (Figure 5a was part of the recording). (a) Histogram of GABA effective mean open times, mean ± s.d. = 32.6 ± 41.8 ms, 2 ms binwidths. (b) GABA effective closed times, mean ± s.d. = 221 ± 252 ms, 20 ms binwidths. n = 471, ordinate scale: frequency (%).

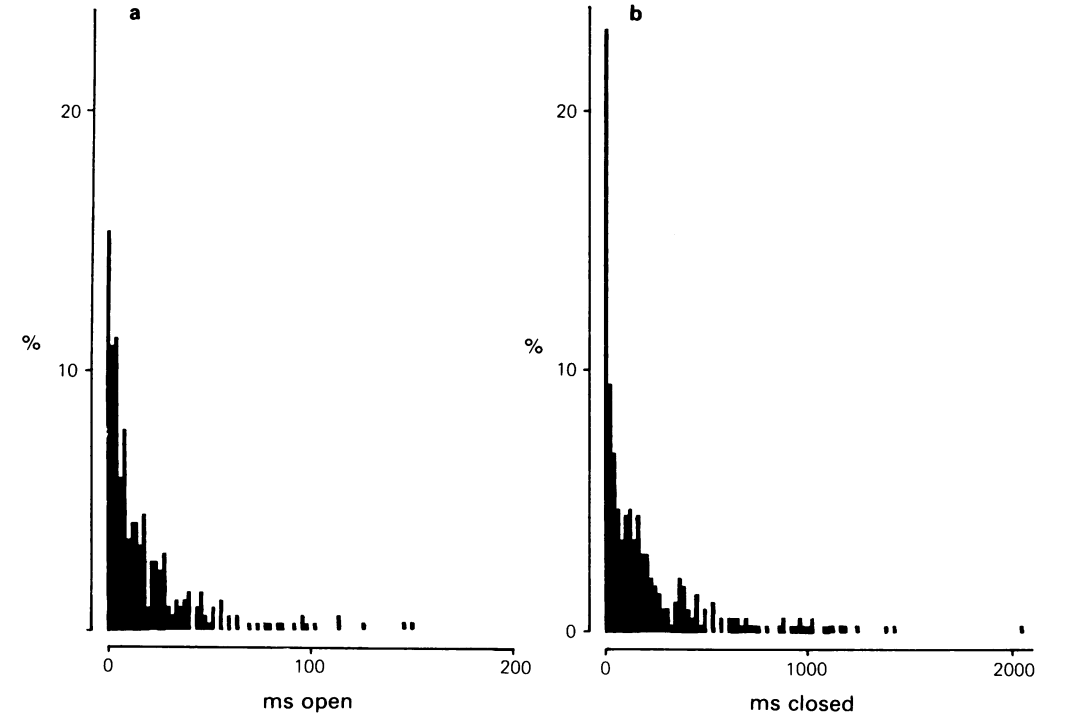

Figure 7 Distributions of piperazine open and closed times. A cell-attached patch with piperazine 500 μM as agonist and transpatch potential of − 75 mV was used (Figure 5b was part of the recording). (a) Histogram of piperazine channel effective open times, mean ± s.d. = 18.7 ± 4.0 ms, 2 ms binwidths. (b) Histogram of piperazine channel effective closed times, mean ± s.d. = 258 + 664 ms, 20 ms binwidths. n = 337, ordinate scale: frequency (%).

13.8 ± 2.4 ms, (mean ± s.e., $n = 4$). Similar results with the same concentrations of agonists were obtained with outside-out patches.

Effect of membrane potential on open time

The effect of membrane potential on channel open times for 3–4 μM GABA and 500 μM piperazine was examined using cell-attached patches. It was found for GABA, using the available observations between − 100 mV and − 40 mV, that there was no significant correlation between the membrane potential and mean channel open times ($r = 0.26, n = 12, P < 0.05$). It was also found for piperazine that there was no significant correlation using the observations between − 150 mV and − 75 mV ($r = 0.6$, $n = 10$, $P < 0.05$). These results suggest that the channel open times were not appreciably voltage sensitive.

The distribution of channel open times

The histograms of Figures 6a and 7a illustrate typical distributions of channel open times for GABA (3 μM) and piperazine (500 μM) recorded under the same patch potential of − 75 mV. It was found that these distributions could not be described with a single exponential but were best described by a double exponential. Table 1 summarises the values of the parameters estimated for these double exponentials. In the experiment with GABA as the agonist the fast component had a mean value of 2.1 ms while the slow component had a mean value of 46 ms. For piperazine the mean value of the fast component was 7.6 ms while the mean value of the slow component was 28 ms. After the double exponential had been fitted it was possible to estimate the relative number of fast openings and slow openings for both GABA and piperazine. It can be seen from Table 1 that 36% GABA and 47% piperazine openings were of the fast type while the remainder were of the slow type. As a consequence the brief openings for GABA carried only 3% of the total current but for piperazine 19% of the current was carried by the brief openings. During both of these recordings no simultaneous openings were observed. Simultaneous double openings would be expected if there were two channel types activated by the GABA agonists. The lack of simultaneous openings then implies that there are at least two open channel states rather than two separate channel populations. (Colquhoun & Hawkes, 1981; 1982). Similar observations have been made with acetylcholine channels (Colquhoun & Sakmann, 1981) and glutamate channels (Cull-Candy & Parker, 1982). Dudel (1979) has observed two components in noise analysis and relaxation studies for GABA activated channels, while Jackson *et al.* (1982) have observed a double exponential in the distribution of GABA channel open times obtained from cultured mouse spinal cord cells.

The distribution of channel closed times

In both cell-attached and outside-out patches with low concentrations of GABA or piperazine as the agonist, opening often occurred in bursts, see for example Figure 2 and 5. The existence of this bursting behaviour was due to brief (1–6 ms) closed periods occurring in sequence between brief or long openings. These brief closings gave rise to the fast components of the exponentials which were required to describe the distribution of the channel closed times (Figures 6b, 7b and Table 2). When the length of the burst was defined

Table 1 Estimates of parameters of open probability density functions

Open distributions	*No of components*	*means* ± s.e. (ms)		
Low agonist concentration	m	U_1	U_2	K_1
3 μM GABA	2	2.1 ± 0.6	46 ± 5	0.36 ± 0.05
500 μM piperazine	2	7.6 ± 3.6	28 ± 6	0.47 ± 0.17
High agonist concentration	m	U_1	U_2	K_1
20 μM GABA	2	4.0 ± 1.4	101 ± 17	0.09 ± 0.04
1 mM piperazine	1	—	53 ± 2	0

The probability density functions of channel open times were described by a mixture of m exponentials.

$$f(T) = \sum_{i=1}^{m} \frac{Ki}{Ui} e^{-T/Ui}$$

, where the i th exponential has a mean value Ui ms and is present in the proportion K_i ($1 > K_i > 0$). The best estimates ± s.e. for the parameters − 75 mV, cell-attached patches, typical K_i & U_i from single patch recordings.

T is ms and $\sum_{i=1}^{m} K_i = 1$

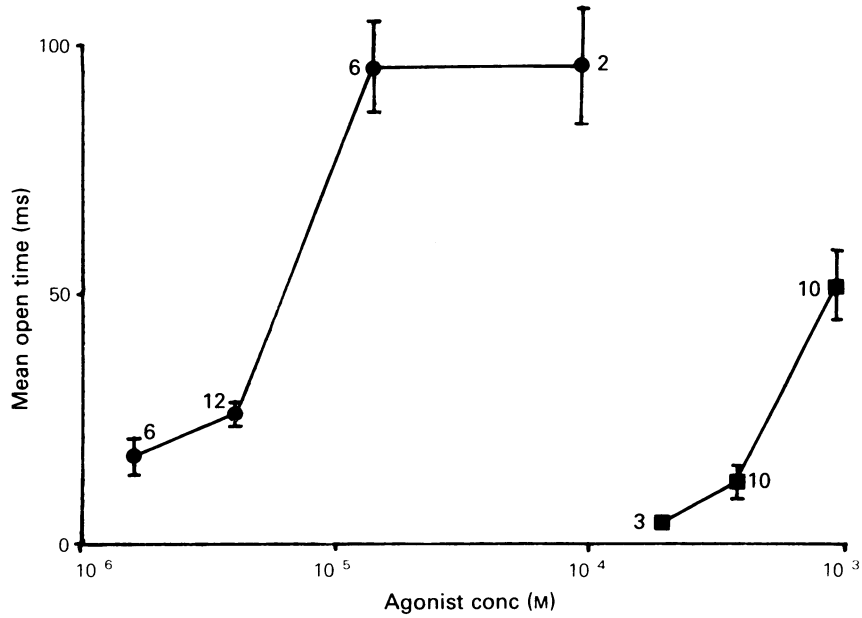

Figure 8 Effective mean open time-agonist concentration relationship. Ordinate scale: effective mean channel open time. Abscissa scale: agonist concentration. GABA (●); piperazine (■). Cell-attached patches were used with transpatch potentials between − 50 and − 100 mV. The numbers of recordings, each with 70–900 openings, together with the standard error bars are shown.

as the duration of a single opening or sequence of openings separated by closings less than 7.5 ms it was found that the mean burst length depended on the nature of the agonist. The mean burst duration for 3 μM GABA was always longer (39 ms in the experiment illustrated in Figure 5) than for 500 μM piperazine (24 ms in the experiment in Figure 5). This bursting behaviour was observed in outside-out patches soon after the bath-application of low agonist concentrations (e.g. Figures 2 and 10) and this suggests that the phenomenon is not due to desensitization (Sakmann *et al.*, 1980). Another possible explanation for the bursting behaviour could have been that the agonists were acting in addition as channel blockers (Neher & Steinbach, 1978). Agonist channel block was thought unlikely since it was found that the effective mean channel open time actually increased rather than decreased with agonist concentration (Figures 8, 9 and 10). A channel block mechanism would be expected to produce a decrease in open time with an increase in agonist concentration. It was also found that the mean channel open times were not appreciably voltage sensitive, unlike a channel block mechanism. The distributions of the closed times at

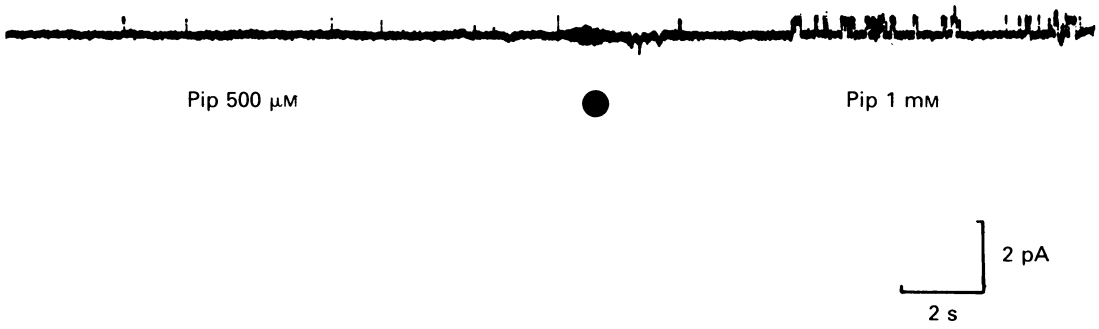

Figure 9 Effect of increasing agonist (piperazine) concentration on an outside-out patch. An outside-out patch recording is shown with a transpatch potential of + 50 mV. At the artefact marked (●) the concentration of piperazine was increased from 500 μM to 1 mM. The probability of the channel being open increased from less than 0.1 to 0.3. There was also an associated increase in effective mean channel open time from 12 ms to 38 ms. The channel activity became quiet after 1 min in the 1 mM piperazine, presumably due to desensitization (not shown).

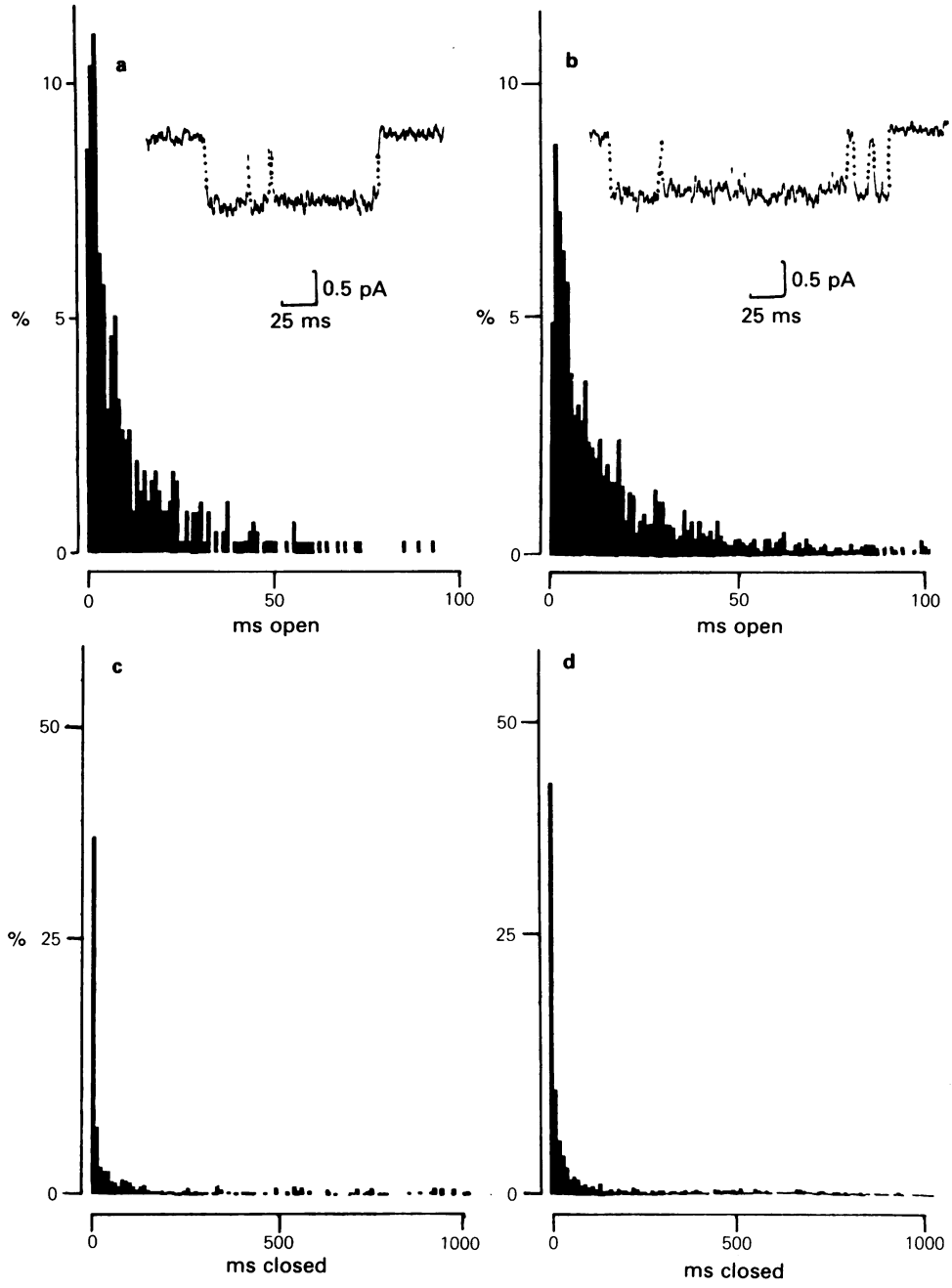

Figure 10 Histograms of the open and closed time distributions at two GABA concentrations: isolated outside-out patch − 75 mV (inset, openings inward currents shown downwards, retouched), temperature 22°C. (a) GABA 2.5 μM; sample recording and histogram of open times; 1 ms binwidths; $n = 452$; ordinate scale: frequency percentage; effective mean open time ± s.d. 14.4 ± 19.5 ms. (b) GABA 4.5 μM; sample recording and histogram of open times; 1 ms binwidths; $n = 1532$; ordinate scale: frequency percentage; effective mean open time ± s.d. 24.4 ± 49.7 ms; note the decrease in the proportion of fast openings. (c) GABA 2.5 μM; histogram of closed times; 10 ms binwidths; $n = 452$; ordinate scale frequency percentage; mean closed time ± s.d. 535.1 ± 1044.7 ms. (d) GABA 4.5 μM; histogram of closed times; 10 ms binwidths; $n = 1532$; ordinate scale frequency percentage; mean closed time ± s.d. 281.2 ± 952.4 ms; note the increase in the proportion of brief closings which was associated with a mean increase in the number of rapid closings per burst from 0.64 to 1.4. No desensitization was evident during the application of the higher agonist concentration.

low agonist concentrations were best described by up to three exponentials. The second component in the distribution was due to the tendency of bursts to be separated by longer closed periods (5–150 ms) giving rise to clusters. The third component was due to much longer closed periods which separated the clusters. Table 2 shows the values estimated for the parameters of the exponentials used to describe the distributions of closed times for the experiments illustrated in Figure 5, 6b and 7b. These observations suggest that there are up to three closed states at these agonist concentrations.

Effect of increased agonist concentration

Figure 9 illustrates with an outside-out patch recording effects of increasing the agonist concentration from 500 μM piperazine to 1 mM piperazine. In this experiment the probability of the channel being open increased from less than 0.1 with 500 μM piperazine to 0.3 with 1 mM piperazine. This was associated with an increase in the effective mean channel open time from 12 ms to 38 ms. The number of openings per bursts increased from about 1 to a mean of 3.2; the burst duration increased from a mean of 12 ms to a mean of 122 ms. After a period of 30 s at this higher concentration of piperazine the channel became quiet, presumably due to desensitization (not shown).

Figure 10 illustrates some representative recordings and the distributions of open and closed times from another outside-out patch experiment where the GABA concentration was increased from 2.5 μM to 4.5 μM. The probability of the channel being open increased from 0.03 to 0.09; the effective mean channel open time increased from 14 ms to 24 ms. Comparison of Figure 10a and b shows that the proportion of brief openings declined with increased agonist concentration. In contrast the proportion of brief closings increased (Figures 10c and d). The number of brief closings per burst increased from a mean of 0.64 to 1.4; the mean burst duration increased from 24 ms to 58 ms. When the distributions of the open times were described by the sum of two exponentials it was found that: the proportion of brief openings declined from 48% to 23% (both with a mean of 4.0 ms); in contrast the proportion of longer openings increased from 52% (mean 22 ms) to 77% (mean 46 ms). When the distributions of the closed times were described by the sum of exponentials it was found that: the proportion of brief closings forming bursts increased from 39% (mean 4.5 ms) to 58% (mean 3 ms); the second closed component separating the bursts and forming clusters became shorter and reduced from a mean of 50 ms (20% of closings) to 28 ms (19% of closings); the third component separating clusters also became shorter and decreased from a mean of 1,180 ms (41% of closings) to 740 ms (23% of closings).

Table 2 Estimates of parameters of closed probability density functions

Closed distributions	*No. of components*	*means ± s.e. (ms)*						
		U_1	U_2	U_3	U_4	K_1	K_2	K_3
Low agonist concentration	m							
3 μM GABA	3	1.6 ± 0.5	40 ± 20	1320 ± 130		0.13 ± 0.06	0.07 ± 0.03	
500 μM piperazine	3	1.8 ± 0.6	130 ± 30	510 ± 100		0.20 ± 0.03	0.51 ± 0.09	
High agonist concentration	m							
20 μM GABA	4	5.6 ± 1.1	34 ± 14	310 ± 170	1950 ± 60	0.49 ± 0.07	0.19 ± 0.07	0.13 ± 0.04
1 mM piperazine	4	2.7 ± 0.2	13 ± 2	230 ± 80	2760 ± 70	0.69 ± 0.04	0.23 ± 0.04	0.04 ± 0.01

The probability density functions of channel closed times were described by a mixture of m exponentials.

$$f(T) = \sum_{i=1}^{m} \frac{Ki}{Ui} e^{-T/Ui}$$

, where the i th exponential has a mean value U_i ms and is present in the proportion K_i ($1 > K_i > 0$).
The best estimates ± s.e. for the parameters − 75 mV, cell-attached patches, typical K_i & U_i from single patch recordings.

T is ms and $\sum_{i=1}^{m} K_i = 1$

The effect of agonist concentration over a wider range was examined using cell-attached patches. Figures 8 and 12c and d show the effect of concentration on effective mean open times for GABA and piperazine. It can be seen that the log-dose response relationship for piperazine resembles that of GABA but it is shifted along the concentration axis. Tables 1 and 2 show some typical probability density functions estimated for channel open and closed times at 20 μM GABA and 1 mM piperazine; part of the recordings are shown in Figure 12. It can be seen as before that there is a decrease in the proportion of brief openings and an increase in the proportion of brief closings with increasing agonist concentration. For example, at 3 μM GABA, 36% of the openings were of the brief type but at 20 μM, only 9% were of the brief type (Table 1); at 3 μM GABA, 13% of the closings were of the brief type while at 20 μM, 49% were of the brief type (Table 2). A similar change in the proportion of brief events were also seen with piperazine (Tables 1 and 2). Increases in channel open time with an increase in ligand concentration have not been observed with acetylcholine (Sakmann *et al.*, 1980) but they have been observed for the glutamate receptor (Gration *et al.*, 1981) and for Ca^{2+} activated K^+ channels (Barret *et al.*, 1982).

Desensitization

Desensitization was often seen after prolonged application of higher agonist concentrations. It was interpreted as the cause of the decline, sometimes abrupt, in the probability of the channel being open. It was associated with long closed periods (seconds) between normal periods of activity giving rise to grouping of channel activity. This behaviour is illustrated in Figure 11 with an outside-out patch following application of 4.6 μM GABA. Following channel activation for 60 s there was a long quiescent period. After 90 s channel opening recommenced and contin-

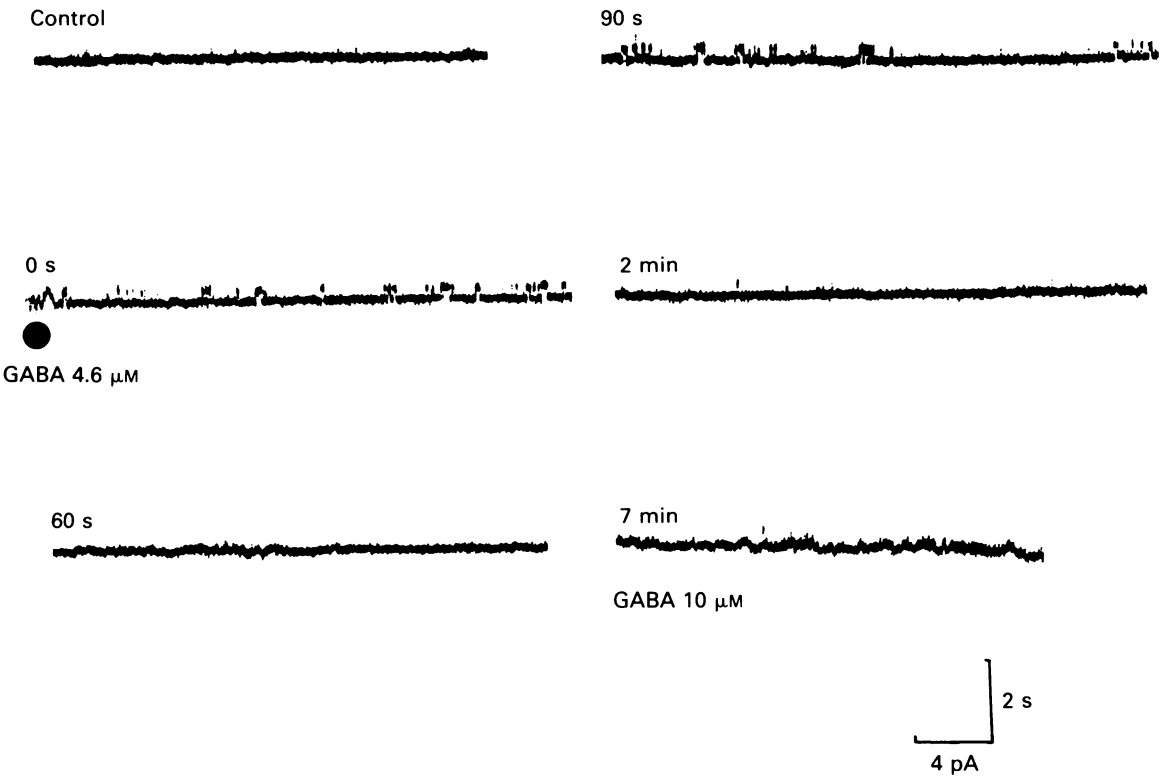

Figure 11 Desensitization: an outside-out patch recording is shown, transpatch potential + 40 mV. During the control period no channel openings were observed. At the artefact marked (●) GABA 4.6 μM was added to the bath and produced channel openings (outward currents, upward). After 60 s a quiescent period followed and after a further 90 s channel opening recommenced. The channel openings then continued until a period marked 2 min when the recordings again became very quiet. During this period marked 2 min, only two openings were observed. The preparation remained quiet and after a period of 7 min a concentration of GABA 10 μM was added to the bath and produced no increase in the probability of the channel being open.

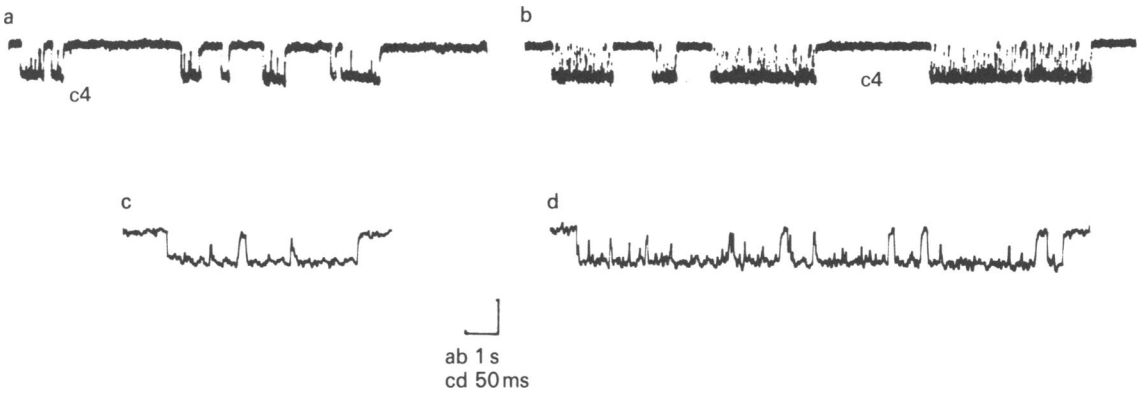

Figure 12 Single channel currents recorded using high agonist concentrations, cell-attached patches, transpatch potential − 75 mV. (a) GABA 20 μM as agonist. (b) Piperazine 1 mM as agonist. Note the presence of the long closed periods (c4) seen with the slow recording speeds. (c) Part of the recording of (a) but at a higher time resolution; GABA effective mean open time 95 ms. (d) Part of recording of (b) but at higher time resolution; piperazine effective mean open time 54 ms.

ued until 2 min after application when the recording again became quiet. Channel activity was then not further stimulated by the addition of a higher (10 μM) concentration of GABA. Similar very long closed periods between opening or grouping activity were also seen with cell-attached patches and with piperazine as the agonist. Table 2 shows the values for the probability density functions which were used to best describe the distribution of the closed periods with 20 μM GABA and 1 mM piperazine as agonists with cell-attached patches at − 75 mV; parts of the recordings used for the analysis are shown in Figure 12. Up to four exponentials were required to best describe the distributions suggesting that there are up to four closed mainstates at higher agonist concentrations. The longest component was not present at lower concentrations and presumably corresponded to the desensitized state. Figures 12a and b show examples of the grouping of clusters which was observed using 20 μM GABA and 1 mM piperazine as agonist. Note the appearance of long closed periods lasting several seconds.

Discussion

The primary aim of this series of experiments was to examine the behaviour of the single channel currents produced by activation of membrane receptors with GABA and piperazine. It had already been shown that these agonists will activate extra-synaptic receptors found on the bag region of the *Ascaris* muscle cell and that they give rise to an increase in Cl^- conductance (Martin, 1982).

Distribution of channel open times

The distribution of the channel open times produced by piperazine and GABA were usually described by the sum of two exponentials. At least 35% of the openings were found, at low agonist concentrations, to be due to the brief openings. The remainder were estimated to be due to the slower openings. Double exponential distributions have been implied or observed in single channel studies with acetylcholine (Colquhoun & Sakmann, 1981), glutamate (Cull-Candy & Parker, 1982), calcium activated potassium channels (Barret *et al.*, 1982) and GABA channels in cultured cells (Jackson *et al.*, 1982). The lack of simultaneous double openings observed suggests that the double exponential distribution cannot be explained by more than one channel population but implies the existence of more than one open channel mainstate. It has been suggested (Colquhoun & Sakmann, 1981; Cull-Candy & Parker, 1982) that the brief openings correspond to openings of receptor channels occupied only by one agonist molecule and that the longer opening state corresponds to receptor-operated channels occupied by two agonist molecules. These observations on the channel open times are inconsistent with a simple two step KM model (Del Castillo & Katz, 1957; Katz & Miledi, 1972), which involves the binding of a single agonist molecule for channel opening.

Increases in agonist concentration in this study were associated with increases in effective mean open time and decreases in the proportion of brief openings. One possibility is that this might be due to the failure to resolve brief intervening closings (Neher, 1983) due to

the limited frequency response of the amplifier; the observations in this paper were carried out under conditions which did not always permit good resolution of events less than 1 ms. However, it is pointed out that Gration *et al.* (1981) have previously observed concentration-dependent increases in mean open time of glutamate-activated channels.

One attractive model of agonist action in which two agonist molecules can combine to two subunits of the receptor-channel complex to produce opening is the Monod-Wyman-Changeux model (Colquhoun & Hawkes, 1977; 1982). It can account by a plausible physical mechanism for: the changes in mean open time observed in these experiments with agonist concentration: the double exponential distribution of the open times and the change in the proportion of fast and slow openings: the greater than linear relationship between the probability of the channel being open and the agonist concentration.

The distribution of channel closed times

The distribution of channel closed times for piperazine and GABA could not be described by a single exponential but required up to three exponentials with low agonist concentrations; the brief components corresponding to the rapid closings during bursts. Channel block by the agonist was thought unlikely, because of the observed increase in the mean open times associated with an increase in agonist concentration, and no voltage sensitivity (Neher & Steinbach, 1978). One explanation of this phenomenon is the 'Nachschlag' effect (Colquhoun & Sakmann, 1981). This is the isomerization of the channel to the open configuration after closing without dissociation of agonist molecules. The existence of the three exponentials in the probability density functions which best describe the distributions of the closed times at low agonist concentrations suggest the presence of three closed states.

At high agonist concentrations and after an initial period of a high probability of channel opening, long closed periods appeared separating openings into groupings of clusters. The distribution of channel closed times at these higher agonist concentrations were best described by up to four exponentials suggesting the existence of at least four closed states. The extra component at the higher concentrations was interpreted as due to the appearance of the desensitized state. Sakmann *et al.* (1980) have noted long closed states at high agonist concentrations and interpreted them as due to desensitization. However, desensitization has not been noted before during briefer bath applications of GABA and piperazine (Martin, 1980; 1982).

Differences between piperazine and GABA

Piperazine is some 10 to 100 times less potent than GABA in the *Ascaris* preparation (Martin, 1982). The mainstate channel conductances of both agonists were similar so that any conductance differences could not explain the magnitude of the observed differences in potency. This difference in potency may be explained by the fact that piperazine required a higher concentration to achieve a similar opening rate to GABA. In addition, the effective mean open time for piperazine was generally shorter than that of GABA. Thus, for example, 3.5 μM GABA in one typical experiment produced a mean opening rate of 3.9 s^{-1} with an effective mean open time of 33 ms and a probability of the channel being open of 0.14. On the other hand, another typical experiment using 500 μM piperazine as the agonist produced a channel opening rate of 3.6 s^{-1}, and an effective mean open time of 19 ms giving a probability of 0.08 for the channel being open. Lambert *et al.* (1983) have already suggested that in general, less potent agonists have shorter open channel lifetimes.

The observation that piperazine is an agonist suggests that an adequate interaction of the amine moiety with the presumed anionic sub-site of the receptor is enough to permit channel opening. Piperazine has a heterocyclic ring structure and lacks a carboxyl group like that of GABA. The fact that the effective mean open time of GABA is generally longer than that of piperazine suggests that some interaction between the carboxyl group of GABA and the receptor may be important in delaying channel closure in addition to facilitating channel opening. It is interesting that Auerbach *et al.* (1983) have reported analogous results with acetylcholine agonists. They have suggested that the continued presence of the agonist in the transmitter binding site affects the stability of the open channel.

It was found in these studies that piperazine mimics the action of GABA in that they are both able to open channels of the same mainstate conductance. Evidence was found for both compounds producing two open mainstates and up to three closed states as well as an additional desensitized state. The lower potency of piperazine could be accounted for if higher concentrations of this drug were required to produce channel openings which generally had shorter mean open times.

The density of extra-synaptic GABA receptors

A peak current of about 20 nA can be generated by iontophoretic application of GABA to the bag under voltage clamp with a driving force of 30 mV (Martin,

1982). Thus each channel would carry a current of 0.66 pA (G = 22 pS) and require the activation of about 29,000 receptors. The bag membrane surface exposed to GABA is approximately 70,000 μm^2 and this suggests an average area occupied by each receptor of about 2.4 μm^2. On this basis each typical cell-attached patch of 5–10 μm^2 (Sakmann & Neher, 1983) would be expected to contain approximately 2–4 receptor-operated channels. However, only about 1 in 7 of the patches created in these experiments showed any (one or more channels per patch) recordable GABA-activated channel activity. The explanation for the much lower observed success rate is not known. One possibility is that desensitization might have occurred during patch formation.

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