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GABA Concentration Sets the Conductance of Delayed $GABA_A$ Channels in Outside-out Patches from Rat Hippocampal Neurons

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Abstract. $GABA_A$ channels were activated by GABA in outside-out patches from rat cultured hippocampal neurons. They were blocked by bicuculline and potentiated by diazepam. In 109 of 190 outside-out patches, no channels were active before exposure to GABA (silent patches). The other 81 patches showed spontaneous channel activity. In patches containing spontaneous channel activity, rapid application of GABA rapidly activated channels. In 93 of the silent patches, channels could be activated by GABA but only after a delay that was sometimes as long as 10 minutes. The maximum channel conductance of the channels activated after a delay increased with GABA concentration from less than 10 pS (0.5 μM GABA) to more than 100 pS (10 mM GABA). Fitting the data with a Hill-type equation gave an EC_{50} value of 33 μM and a Hill coefficient of 0.6. The channels showed outward rectification and were chloride selective. In the presence of 1 μM diazepam, the GABA EC_{50} decreased to 0.2 μM but the maximum conductance was unchanged. Diazepam decreased the average latency for channel opening. Bicuculline, a GABA antagonist, caused a concentration-dependent decrease in channel conductance. In channels activated with 100 μM GABA the bicuculline IC_{50} was 19 μM. The effect of GABA on channel conductance shows that the role of the ligand in $GABA_A$ receptor channel function is more complex than previously thought.

Key words: $GABA_A$ channels — Ligand-gated — Inhibition — $GABA_A$ receptors — Anaesthetics — Benzodiazepines

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Introduction

The conductance of an ion channel is generally regarded as a defining property of the channel that depends on its 3-dimensional structure and, in particular, the residues lining the channel. The ubiquitous, inhibitory $GABA_A$ receptor is thought to be a heteropentamer with two GABA binding sites and the channel is assumed to be formed by the highly homologous TM2 segments of the five subunits (Stephenson, 1995; Barnard et al., 1998; Miyazawa, 1999). Such a structure might be expected to form a channel of reasonably constant conductance. It is well established, however, that the conductance state of a $GABA_A$ receptor can vary and prominent subconductance states are a characteristic feature of the receptor (for reviews *see* Mathers, 1991; Sivilotti & Nistri, 1991; MacDonald & Olsen, 1994). The maximum conductance has been shown to vary among different neuronal preparations (Hamill, Bormann & Sakmann, 1983; Gray & Johnston, 1985; Smith, Zorec & McBurney, 1989; Mathers, 1991) and work on reconstituted receptors indicates that the maximum conductance observed may vary with the subunit composition of the receptors (Mathers, 1991; Rabow, Russek & Farb, 1996). Considering the number of $GABA_A$ subunits that can form pentamers (19 subunits, Barnard et al., 1998), a variety of single-channel conductances might not be unexpected.

We have suggested previously that the conductance of $GABA_A$ receptors in dentate gyrus granule cells and CA1 pyramidal neurons in the rat hippocampal slice preparation may be affected by the GABA concentration (Birnir, Everitt & Gage, 1994; Birnir et al., 2000a). A conductance varying with ligand concentration is not a unique feature of $GABA_A$ receptors. Single-channel conductance has been correlated with agonist concentration both in cyclic nucleotide-gated channels (Ruiz and

Karpen, 1997) and in AMPA receptors (Rosenmund, Stern-Bach & Stevens, 1998; Smith & Howe, 2000). In some ATP-gated channels, the ion-selectivity can change during ligand application (Surprenant et al., 1996; Khakh & Lester, 1999), another example of how dynamic the functional properties of channels can be. We have now examined in greater detail in outside-out patches from cultured hippocampal neurons the relationship between channel conductance and agonist concentration. In patches not containing any channel activity prior to GABA application ("silent patches") we have found a direct correlation between GABA concentration and the maximum conductance of channels activated by GABA. A preliminary account of some of these observations has appeared elsewhere (Birnir et al., 1998, 1999).

Materials and Methods

Neurons used in the experiments were dissociated from hippocampal slices from newborn rats (<18 hr old) and maintained in culture for 8 to 24 days using techniques described previously (Curmi et al., 1993). Experiments were done at room temperature (20–24°C) on outside-out patches except where otherwise stated. Channels were activated either by switching the solution flowing through the bath to a solution containing GABA (flow rate 4 ml/min, bath volume 0.4 ml) or by flowing a solution containing GABA through a narrow tube superfusing the patch. Using this latter method, the rate of solution exchange for an open tip electrode was less than 1 msec (Birnir et al., 1995). In most experiments, the pipette potential was +40 mV since $GABA_A$ channels are more active at depolarized than at hyperpolarized potentials (Weiss, Barnes & Hablitz, 1988; Birnir et al., 1994). Bath solution contained (mM): NaCl 135, KCl 3, $CaCl_2$ 2, $MgCl_2$ 2, TES (*N*-tris(hydroxymethyl)methyl-2-amino ethane sulphonic acid) 10, pH 7.4. In some experiments in which the reversal potential of currents was examined, 116 mM NaCl was replaced with 116 mM Na gluconate. Pipette solution contained (mM): NaCl or choline Cl 141, KCl 0.3, $CaCl_2$ 0.5, $MgCl_2$ 2, EGTA 5, TES 10, pH 7.4. In some experiments, the pipette also contained 4 mM ATP but this had no detectable effect. γ-Aminobutyric acid (GABA, Sigma, St. Louis, MO) and bicuculline methiodide (Sigma) were dissolved in the bath solution. Diazepam (Hoffman-La Roche, Nutley, NJ) was first dissolved in DMSO as described by Eghbali et al. (1997).

Conventional patch-clamp techniques were used when establishing a gigaseal and forming patches (Hamill et al., 1981). Pipettes were made from borosilicate glass (Clark Electromedical, Reading, UK), coated with Sylgard (Dow Corning, Midland, MI) and fire-polished. Their resistance ranged from 10 to 20 MΩ. Currents were recorded using an Axopatch 1C current-to-voltage converter (Axon Instruments, Burlingame, CA), filtered at 5 kHz, digitized at 44 kHz using a pulse code modulator (Sony PCM 501, Sony, Tokyo, Japan) and stored on videotape. The currents were played back from the videotape through the Sony PCM and digitized at a frequency of 10 kHz using a Tecmar analog-to-digital converter interfaced with an IBM-compatible PC. The currents were then digitally filtered (Gaussian filter) at 5 (or occasionally 2) kHz and analyzed using a computer program called CHANNEL2 written by Michael Smith (JCSMR, A.N.U., Canberra). The amplitude of currents was measured either from all-points current-amplitude probability histograms or from direct measurements of the amplitude of individual currents filtered at 5 kHz. As the channels generally displayed frequent subconductance states, we use the terms "single-channel current" or "single-channel conductance" for the maximum current or conductance levels observed that showed direct transitions to or from the zero current level or conductance levels more frequently than would be expected from the opening and closing of two or more independent channels. Lower conductances are called "subconductance" states. The mean current was measured as the average of the deviations of all data points from zero (the middle of the baseline current) during periods of 20 sec to 1 min. The average open probability of channels was measured from opening and closing transitions detected by setting threshold levels just above the baseline noise. For illustrative purposes current traces were sometimes filtered at 2 kHz unless this distorted current levels recorded with a 5 kHz filter (The filter frequency is specified in the figure legends). A correction was made for junction potentials using JPCalc (P.H. Barry, U.N.S.W., Sydney) where appropriate. Data are expressed as means ± SEM (n = number of patches).

Results

Silent Outside-out Patches

Slowly Activating (*Delayed*) *Channels*

No spontaneous channel activity was detected in 109 of 190 outside-out patches. After application of GABA (0.5 μM to 10 mM), single-channel currents appeared in 93 of the 109 "silent" patches. GABA was either bath-applied or applied by the fast superfusion system to the outside-out patches. In either case, large conductance channels were not usually seen immediately. It often took tens of seconds to minutes until channels with maximal conductance were observed, no matter what GABA concentration was used. In only two patches were channels with maximal conductance seen within a fraction of a second after GABA application. Examples of this variability are shown in Fig. 1. The current traces are from two patches exposed to 100 μM (Fig. 1*A*) and 1 mM GABA (Fig. 1*B*). Neither patch had been previously exposed to GABA. In Fig. 1*A* (pipette potential V_p = +80 mV) there was a delay of about 4.2 min before a 75 pS channel appeared, whereas in Fig. 1*B* (V_p = +40 mV), a maximum conductance channel (65 pS) was observed soon after GABA application. Similar channels were never seen during prolonged periods in patches not exposed to GABA nor could they be activated by simply changing the holding potential.

In the remaining 81 patches, spontaneous single-channel currents were recorded. An example of channel activity in one such patch is shown in Fig. 2*A* (V_p = +40 mV). The patch had spontaneous channel currents of 1.3 pA (32 pS). When 100 μM GABA was applied to the patch, the current increased rapidly to 16.5 pA (413 pS, Fig. 2*Aa*). The rise of the current was fast and had reached 80% of the peak current amplitude in 600 μsec (Fig. 2*Ab*). A detailed description of the characteristics of the spontaneous channels has appeared elsewhere (Birnir et al., 2000b). A rapid response to 100 μM or 10

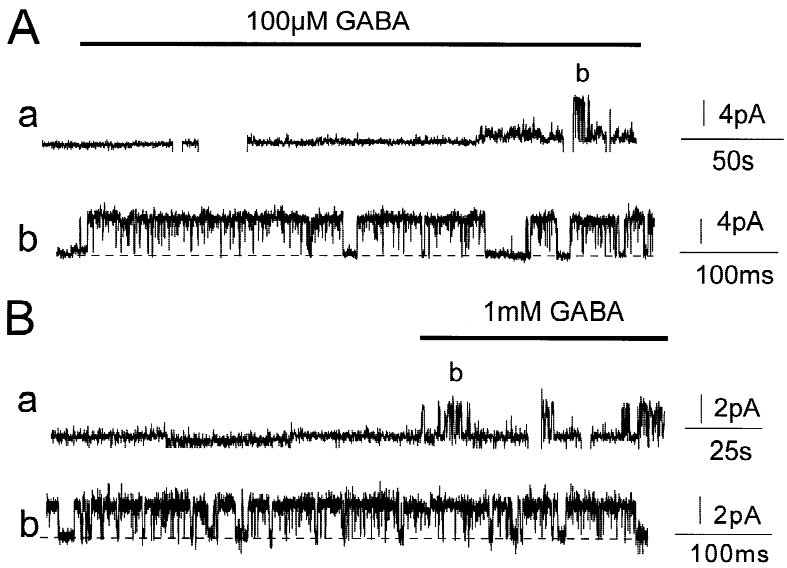

Fig. 1. Delayed activation of channels by GABA in silent outside-out patches. GABA was applied to silent outside-out patches. The breaks or depressions in the current records indicate periods when the potential was briefly changed and the horizontal bars denote exposure to GABA. Currents were filtered at 5 kHz. (*A*) 100 μM GABA was applied and a 75 pS channel was activated at *b* (V_p = +80 mV). The compressed trace in *Aa* is 4.8 min. (*B*) 1 mM GABA was applied and a 65 pS channel was activated at *b* (V_p = +40 mV). The compressed trace in *Ba* is 3.3 min. Calibrations for each trace are shown on the right.

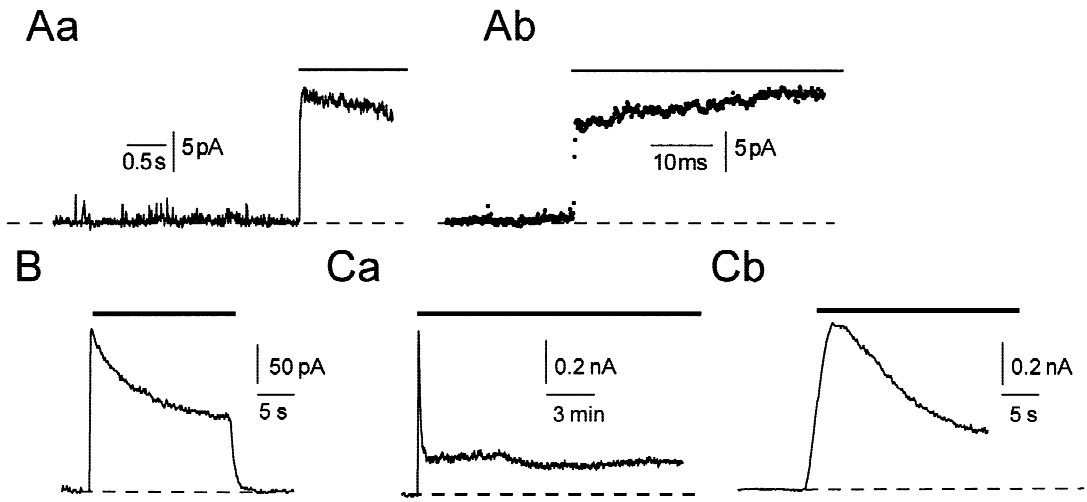

Fig. 2. The response to GABA in patches containing spontaneous channel activity. (*Aa*) Rapid activation of channels by GABA was recorded only if the patch contained spontaneously opening channels. 100 μM GABA was rapidly applied (horizontal bar) to an outside-out patch (V_p = +40 mV) containing spontaneously active, 32 pS channels. (*Ab*). The rising phase of the current shown in *Aa* at a faster time scale. The data points are at 100 μsec intervals. (*B*) Whole-cell current response to 100 μM GABA. (*Ca*) Whole-cell current response from another cell to a prolonged application of 100 μM GABA. The current trace is 18 minutes. (*Cb*) The initial phase of the current trace shown in *Ca* at a faster time scale. GABA was applied for the period of the horizontal bar (V_p = +40 mV) and the dashed line indicates the level of the baseline current.

mM GABA was recorded in five other outside-out patches containing spontaneous channels but never in the silent outside-out patches. Rapid application of GABA gave an immediate increase in whole-cell current in these neurons as illustrated for two different cells in Fig. 2*B* and 2*C* (V_p = +40 mV). The increase in current when GABA was applied to whole cells was similar to that recorded in patches containing the spontaneous channels. Although GABA channels were activated after a delay in the silent outside-out patches from these neurons, we never saw a delayed increase in whole-cell current (*see* Fig. 2*Ca*, 18 min current trace).

The time for first appearance (latency) of maximal conductance channels recorded in 24 silent outside-out patches is plotted against GABA concentration in Fig. 3*A*. It can be seen that the latency varied from patch to patch and there was no clear correlation between latency and GABA concentration. We have reported previously a delayed increase in the conductance of channels activated by low concentrations of GABA in cell-attached patches on neurons in hippocampal slices (Birnir et al., 1994; Birnir et al., 2000a). Similarly, in cell-attached patches on the cultured neurons used in this study, low concentrations of GABA (0.5–5 μM) could activate high

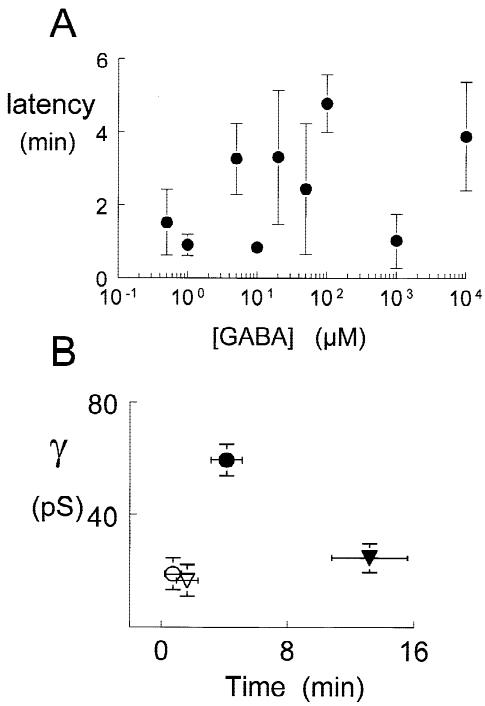

Fig. 3. Latency is independent of GABA concentration in the silent outside-out patches. (*A*) The delay (latency) between GABA application and first appearance of maximum-conductance channels is plotted against GABA concentration. Results shown were obtained from 24 outside-out patches (V_p = +40 mV) and the symbols represent the average ± SEM of three or more measurements. (*B*) In cell-attached but not in outside-out patches, low GABA concentrations can activate high-conductance channels with time. Channels were activated by 0.5 or 5 μM GABA in cell-attached (circles, $-V_p$ = 40, 60 or 80 mV, n = 14) or "silent" outside-out (triangles, V_p = 40 or 60 mV, n = 5) patches. Open symbols show the average initial conductance and filled symbols show the average maximal conductance. For some cell-attached patches, saclofen (50 μM, n = 2; 200 μM, n = 3) was included in the pipette solution to block $GABA_B$ receptors. Symbols represent the average ± SEM.

conductance (>40 pS) channels after a delay (n = 14, Fig. 3*B*). In 5 experiments, the $GABA_B$ receptor antagonist, saclofen (50 μM, n = 2; 200 μM, n = 3), was present in the pipette solution, but the results were similar. Comparable activation of high-conductance channels by low GABA concentrations (n = 5) was never recorded in the silent outside-out patches (Fig. 3*B*) but high-conductance channels were seen with high GABA concentrations (*see below*). In the remainder of this paper we will examine the properties of the delayed channels activated by GABA in the silent, outside-out patches.

We thought it possible that prior exposure to GABA might decrease the latency during subsequent application of GABA. This did not appear to be the case. A current trace from a patch exposed to two concentrations of GABA is shown in Fig. 4*A* (V_p = +40 mV). The patch was first exposed to 5 μM GABA and then 18 min later to 100 μM GABA (Fig. 4*A*). Channels recorded six min after exposure to the 5 μM GABA had a maximum conductance of 17 pS (Fig. 4*A* & *B*) and channel conductance remained the same for the next 12 min. When the patch was exposed to 100 μM GABA large channels with a conductance of 70 pS appeared after about 7.8 min (Fig. 4*A* & *C*). Later, two superimposed currents (Fig. 4*D*) showed that there were at least two channels with conductance of 65 and 70 pS in the patch. During the 17 min exposure to 100 μM GABA, the maximal single-channel conductance remained at 70 pS. In 8 patches where channels were activated by 0.1 to 10 mM GABA concentrations following a previous exposure to a lower GABA concentration, the latency was 3.1 ± 1.2 min. In another 11 patches exposed only to 0.1 to 10 mM GABA, the latency was 4.9 ± 0.8 min. In two other patches, 100 μM GABA was used to activate channels. The GABA was then washed off the patch and re-applied. In both patches, the delay in channel appearance on the second application was similar to the delay following the first application. It therefore appears that pre-exposure to GABA had little if any effect on the latency.

All the single-channel currents recorded, large and small, that appeared after application of GABA reversed at zero millivolts. Substituting choline for sodium, as the main cation in the pipette solution did not change the reversal potential of the currents, indicating the channels were not nonspecific cation channels. In three experiments, in which channels had been activated by 100 μM GABA, the chloride concentration in the bath solution was lowered to 30 mM. The reversal potential changed to +38 ± 1 mV as expected for Cl^--selective channels. Results obtained in one of these experiments are shown in Fig. 5*A*. Initially the patch was exposed to symmetrical chloride solutions (146 mM) and the *IV* relation recorded (filled circles). The reversal potential was close to 0 mV. The chloride concentration in the bath solution was then changed to 30 mM to shift the chloride equilibrium potential to +40 mV and the *IV* relationship recorded again (filled triangles). As expected for a chloride selective channel, the null potential was now close to +40 mV (+37 mV). The *IV* relationship showed outward rectification in both the symmetrical and the asymmetrical chloride solutions. These results confirm that the channels activated by GABA were chloride channels.

The effects of several drugs on the channels further confirmed that they were $GABA_A$ channels. 100 μM bicuculline (n = 6) inhibited and 1 μM diazepam (n = 15) enhanced both large and small conductance channels. These effects are illustrated in Fig. 5*B* & *C*. In Fig. 5*B*, a 62 pS channel activated by 100 μM GABA (*Ba*, V_p = +40 mV) is inhibited by 100 μM bicuculline (*Bb*). In Fig. 5*C*, the conductance of a 21 pS channel activated by

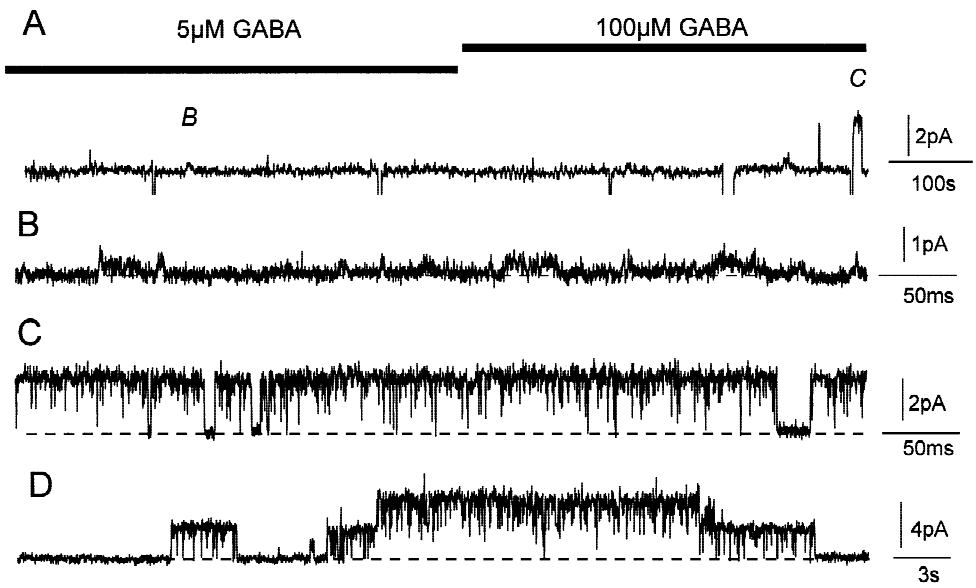

Fig. 4. Delayed activation of channels in silent outside-out patches. (*A*) GABA was applied by fast perfusion to a silent outside-out patch (V_p = +40 mV). The breaks in the current record indicate periods when the potential was briefly changed and the horizontal bars denote exposure to GABA. First 5 μM GABA was applied for 18 min and then 100 μM GABA for 17 min. The current trace in (*A*) shows the last 8.9 min of exposure of the patch to 5 μM GABA and the first 8 min exposure to 100 μM GABA. At both concentrations, maximally conducting channels were activated after a delay (100 μM: 7.8 min, *A* and *C*). In 5 μM and 100 μM GABA, channel conductances were 17 pS (*A, B*) and 70 pS (*A, C*), respectively. In 100 μM GABA, initially there was only one maximally conducting channel but later in the experiment two channels (*D*: 65 and 70 pS) were present. Currents were filtered at 5 kHz. Calibration bars are shown to the right of each trace.

5 μM GABA (*Ca*, V_p = +60 mV) is increased to 52 pS (*Cb*) when 1 μM diazepam is applied to the patch together with the 5 μM GABA. The effects of bicuculline concentration and diazepam on channel conductance are examined in greater detail later.

The Effect of GABA Concentration on Channel Conductance

The maximum conductance of the delayed $GABA_A$ channels was found to be related to the GABA concentration applied. The increase in single-channel conductance caused by raising the GABA concentration is illustrated for 3 patches in Fig. 6*A, B* and *C*. In Fig. 6*A*, the average maximum amplitude of the single-channel currents activated by 0.5 μM GABA was 0.53 pA (13 pS). This small amplitude channel current gave the asymmetry in the all-points histogram on the right of the baseline peak. When the same patch was then exposed to 1 μM GABA, average maximum single-channel current amplitude increased to 0.86 pA (22 pS). This is represented in the all-points histogram by the peak at about 1 pA. The more prominent peak at 0.4 to 0.5 pA is from a more frequent subconductance state. The maximum current amplitude increased further to 1.53 pA (38 pS) when the GABA concentration was raised to 20 μM. Again, the lower peak at 1 pA in the all-points histogram represents a subconductance state. With higher concentrations of GABA, channels with even greater conductance were seen, as illustrated in another patch in Fig. 6*B*. With 5 μM GABA, single-channel currents had an average amplitude of 0.86 pA (22 pS) whereas, when the concentration was raised to 100 μM, average current amplitude was 2.8 pA (70 pS). Some of the largest single-channel currents activated by 10 mM GABA are shown from another patch in Fig. 6*C*. There are 2 independent channels in the patch, with conductances of 85 and 110 pS.

Average maximum single-channel conductance measured at +40 mV in 24 of 26 outside-out patches exposed to a range of GABA concentrations is shown plotted against GABA concentration ([GABA]) in Fig. 7*A*. Conductance was not determined in two patches containing "flickery" channels. The best fit of a Hill-type equation,

$$\gamma = \gamma_{max} \cdot [GABA]^h/([EC_{50}]^h + [GABA]^h), \quad (1)$$

is shown by the solid line and gave a maximum conductance of 113 ± 4 pS, the concentration that gave half-maximal channel conductance, the EC_{50} was 33 ± 6 μM and a Hill coefficient of 0.6 ± 0.1 (r^2 = 0.99). The maximum conductance and the apparent affinity appear to be agonist-dependent as values for channels activated by pentobarbital or propofol differ from those obtained when activated by GABA (Eghbali, Birnir and Gage, unpublished observations).

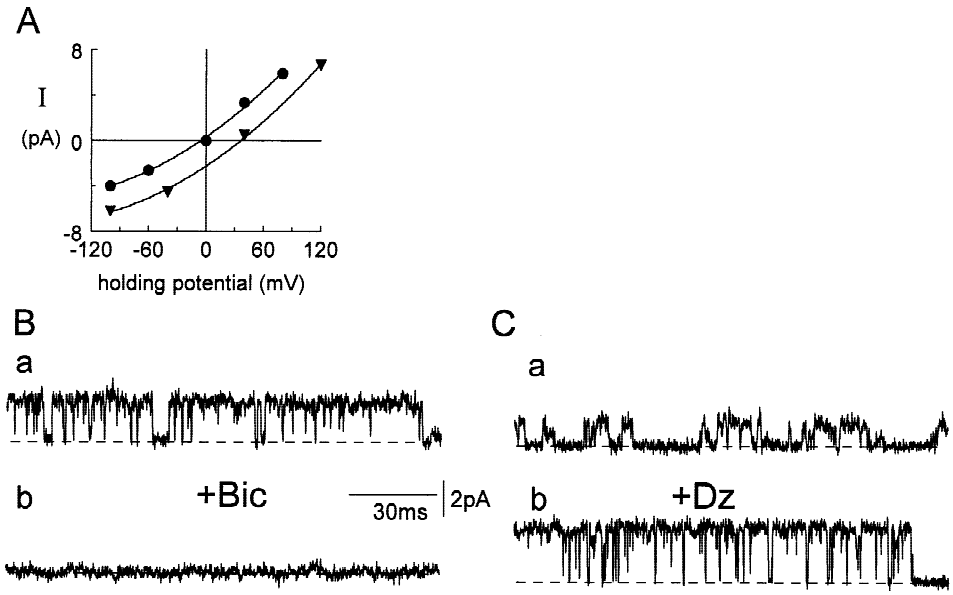

Fig. 5. Characteristics of the channels. (*A*) The GABA-activated currents are chloride selective. Single-channel current amplitudes from one patch were measured by direct measurements of the amplitude of individual openings. Current amplitude is plotted against the holding potential. *Filled circles*: currents recorded in symmetrical Cl^- solutions (146 mM). *Filled triangles*: currents recorded when the bath Cl^- concentration had been lowered to 30 mM. Iso-osmolality was maintained with gluconate. The reversal potential was shifted to +37 mV. (*B*) Bicuculline inhibition. 100 μM GABA activated a 62 pS channel (*Ba*), which was later inhibited by application of 100 μM bicuculline in the presence of 100 μM GABA (*Bb*) (V_p = +40 mV). (*C*) Diazepam enhancement of channel currents. 5 μM GABA activated a 21 pS channel (*Ca*). Channel conductance increased to 52 pS in the presence of 1 μM diazepam (*Cb*) (V_p = +60 mV). Currents were filtered at 5 kHz.

It would be expected that channel-open probability would also increase as the GABA concentration is raised. We therefore examined the effect of GABA concentration on the open probability of the delayed $GABA_A$ channels. Open probability was measured during 20 to 40 sec periods when the channel had reached its maximum conductance. Results obtained from 26 different patches are shown in Fig. 7*B* (V_p = +40 mV). The patches are the same as used in Fig. 7*A* plus two other patches that contained flickery channels. There was an increase in the channel-open probability as the GABA concentration was raised. The best fit of a Hill-type equation (Eq. 1) shown by the solid line gave a maximum channel-open probability of 0.9 ± 0.1, an EC_{50} of 15 ± 9 μM and a Hill coefficient of 0.6 ± 0.2 (r^2 = 0.93). The greater channel-open probability at high concentrations is consistent with reported properties of $GABA_A$ channels (Rabow, Russek & Farb, 1996).

The mean current reflects both single-channel conductance and channel-open probability. The relationship between mean current and GABA concentration is shown in Fig. 7*C* (V_p = +40 mV). The best fit of a Hill-type equation to the data (*see* Eq. 1) shown by the solid line gave a maximum mean current of 4.2 ± 0.5 pA (105 pS), an EC_{50} of 128 ± 68 μM and a Hill coefficient of 0.9 ± 0.3 (r^2 = 0.94). Since the value of the mean current depends on the conductance of the channels (γ, Fig. 7*A*) and the fraction of time they are open during the period of measurement (nPo, Fig. 7*B*), the estimated mean current was calculated from the data in Fig. 7 as $I_{mean} = nP_o \cdot \gamma \cdot (V_m - E_R)$ where nP_o is the probability of the channel being open, γ is the single-channel conductance, V_m is the membrane potential and E_R is the reversal potential of the current. The broken line in Fig. 7*C* represents the product of the Hill fits shown in Fig. 7*A* and *B* and the pipette potential (+40 mV) and correlates well with the measured mean current data. The estimated GABA concentration giving the half-maximal current was 82 μM.

Subconductance States

At any concentration the maximum single-channel conductance varied somewhat between patches e.g. in 10 patches where the channels were activated with 100 μM GABA, it ranged from 60 to 128 pS but the average of all 10 patches was 83 ± 7 pS. In all 10 patches, the currents reversed at V_p = 0 mV. In the same patch, the conductance of channels could vary widely. This is illustrated in Fig. 8 for a patch where the channels had been activated by 100 μM GABA (the five traces in Fig. 8 are continuous). The maximum current was 2.61 pA (65 pS, V_p = +40 mV) but the channel could clearly exist in several conducting states. There was no increase in noise nor any shift in the holding (baseline) current so

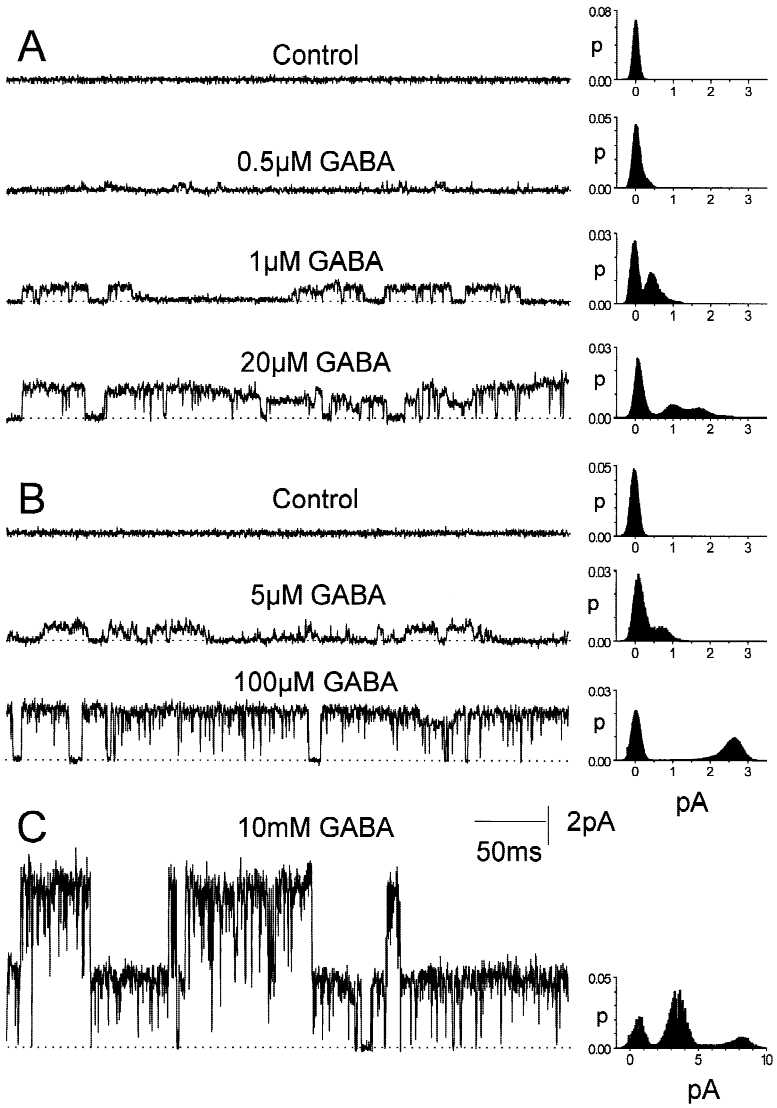

Fig. 6. Properties of channels as a function of GABA concentration. Records in *A*, *B* and *C* are from 3 different outside-out patches exposed to the concentrations of GABA indicated (V_p = +40 mV). The corresponding all-points histograms on the right of each current trace are from 10-sec current records. Currents were digitally filtered at 2 kHz. The calibration bars in *C* apply to all traces.

decreases in amplitude were not due to changes in seal resistance.

Bicuculline Decreases the Conductance of Delayed $GABA_A$ Channels

Bicuculline is regarded as a competitive GABA antagonist at $GABA_A$ receptors (Rabow et al., 1996) and hence should decrease the channel conductance if the probability of GABA binding to the agonist-binding site determines channel conductance. This conclusion is complicated by the observation that spontaneously opening channels can be blocked by high concentration (100 μM) of bicuculline indicating that it is not simply a competitive antagonist (Birnir et al., 2000a & b). We tested the effects of a range of concentrations of bicuculline on channel conductance in 14 patches in which delayed channels had been activated by GABA. In two patches, washing away the bicuculline reversed the effect. Channels were first activated with 100 μM GABA and then the patch was exposed to 100 μM GABA plus bicuculline. It was found that the conductance of channels decreased as the bicuculline concentration was increased, as illustrated in Fig. 9*A* (V_p = +80 mV). Before exposure to bicuculline the maximum, but not the most common, conductance was 62 pS (Fig. 9*A* and *Ba*). In the presence of 5, 20 and 100 μM bicuculline the conductance decreased to 47 pS (Fig. 9*A* & *Bb*), 39 pS (Fig. 9*A* & *Bc*) and 16 pS (Fig. 9*A* & *Bd*), respectively. It can be seen that 100 μM bicuculline did not completely block the channels. In three of the six patches exposed to 100 μM bicuculline, occasional low conductance channel openings were still observed.

The average conductance of channels activated by

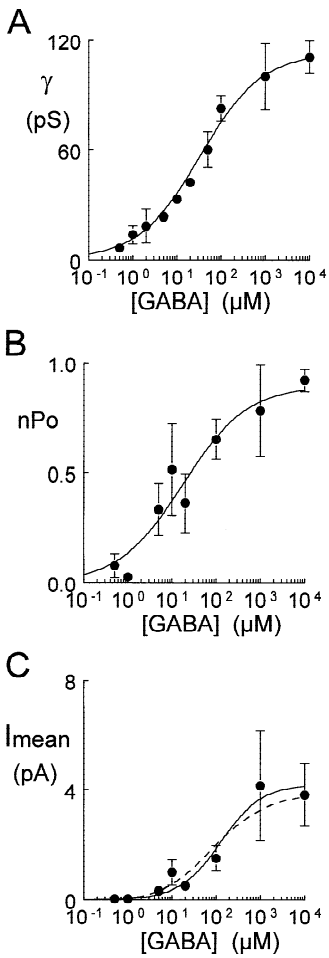

Fig. 7. The conductance of delayed GABA-activated channels increases with GABA concentration. (*A*) Channel maximum conductance (γ, *pS*) is plotted against GABA concentration ([GABA] (μM)). The data, obtained from 24 outside-out patches (V_p = +40 mV), is shown as the average maximal conductance ± SEM if larger than the symbol. The line through the data points is the best fit of equation 1 (*see* Results). (*B*) Open probability of channels (nP_o) is plotted against [GABA]. Results were obtained from 20- to 40-sec current records from 26 outside-out patches during periods of channel activity (V_p = +40 mV). Symbols are the averages ± SEM of three or more measurements. The line through the data points is the best fit of a Hill-type equation (*see* equation 1, Results). (*C*) Mean current (I_{mean}) is plotted against [GABA]. The mean current was determined in 20- to 40-sec records from 26 outside-out patches (V_p = +40 mV). Symbols are the averages ± SEM of three or more measurements. The solid line through the data points is the best fit of a Hill-type equation (*see* equation 1, Results). The broken line is the estimated mean current, $I' = \gamma \cdot n\text{Po} \cdot (V_m - E_r)$, where γ and nP_o were obtained from the fit to the data in Fig. 7*A* & *B*, respectively. V_m is the pipette potential and E_r is the potential for zero current.

100 μM GABA is plotted against bicuculline concentration in Fig. 9*C*. The results were obtained in 14 patches exposed to a range of bicuculline concentrations. The best fit of a Hill-type equation;

$$\gamma = \gamma_{max} \cdot [BIC]^{-h}/([IC_{50}]^{-h} + [BIC]^{-h}) \quad (2)$$

gave a bicuculline IC_{50}, the concentration that reduced the maximal channel conductance to 50%, of 19 ± 2 μM in the presence of 100 μM GABA and a Hill coefficient of 0.8 ± 0.1 (r^2 = 0.99).

Diazepam Increases the Conductance of Delayed $GABA_A$ Channels

We and others have reported previously that benzodiazepines can increase the conductance of $GABA_A$ channels (Eghbali et al., 1997; Guyon et al., 1999). We examined the effect of 1 μM diazepam on delayed single-channel currents evoked by GABA. Results from one patch are shown in Fig. 10*A* (V_p = +40 mV). The top trace (*a*) shows events over a 4 min period. When the patch was exposed to 0.1 μM GABA + 1 μM diazepam the maximum conductance was 39 pS (Fig. 10*Ab*). The maximum conductance then increased to 50 pS and 85 pS in the presence of 0.5 μM GABA + 1 μM diazepam (Fig. 10*Ac*) and 10 μM GABA + 1 μM diazepam (Fig. 10*Ad*), respectively. Application of diazepam alone did not activate channels.

The effect of the GABA concentration on channel conductance in 15 outside-out patches exposed to 1 μM diazepam together with GABA is shown in Fig. 10*B* (V_p = +40 mV). The line through the data points is the fit to the data of a Hill-type equation (*see* Eq.1) with a γ_{max} of 104 ± 4 pS, EC_{50} of 0.20 ± 0.04 μM and a Hill coefficient of 0.7 ± 0.1 (r^2 = 0.99). The broken line shows the relationship obtained in the absence of diazepam (from Fig. 7*A*). Diazepam shifted the conductance GABA-concentration curve to the left to lower GABA concentrations but the maximum conductance was not significantly changed.

The time for first appearance (latency) of maximal conductance channels recorded in the presence of 1 μM diazepam in 10 patches is plotted against GABA concentration in Fig. 10*C*. It can be seen that the latency varied somewhat from patch to patch but in general was shorter than in the presence of GABA alone (*see* Fig. 3*A*).

Discussion

As our aim was to study the influence of GABA concentration on channel conductance, we used outside-out patches so that the GABA concentration could be varied at the extracellular surface of the patch where GABA binding sites are accessible. In 57% of the patches studied, there was no chloride channel activity before patches were exposed to GABA. In 85% of these patches application of GABA elicited channel activity but only after a significant delay.

Delayed Activation

In patches displaying spontaneous channels, channels could be activated rapidly upon GABA application,

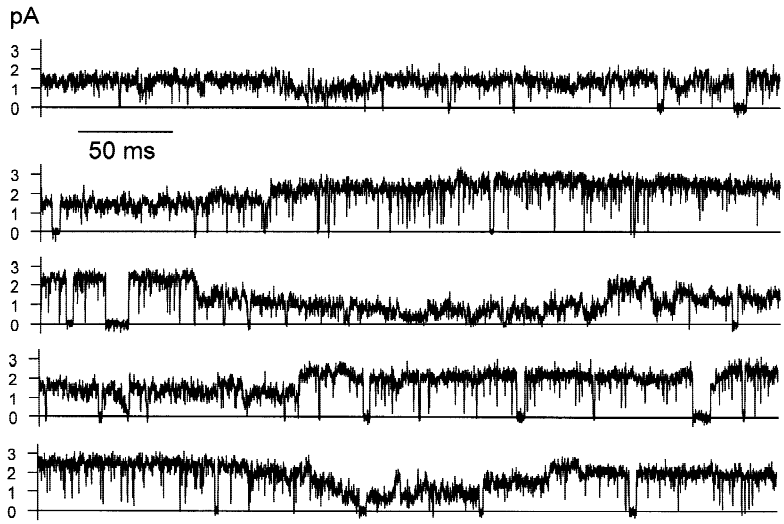

Fig. 8. Variable conductance (substates) of a delayed channel. Currents were activated in a quiet patch with 100 μM GABA. The maximum conductance was 65 pS but the current level varied widely. The duration of each current trace is 400 msec and the five traces are continuous (V_p = +40mV, 5 kHz filter).

whereas in silent patches, application of GABA generally did not cause an immediate appearance of single-channel activity but then, after delays of many seconds to minutes, channel openings suddenly appeared. Similar channels were never seen during prolonged periods in patches not exposed to GABA nor could they be activated by simply changing the holding potential. These delayed channels were clearly associated with $GABA_A$ receptors because they appeared only after exposure to GABA, their amplitude and open probability were related to the GABA concentration, they could be blocked by bicuculline and they were modulated by diazepam. The delay in their appearance is puzzling: it could be due to delayed binding of GABA (e.g., if the GABA binding site were not immediately accessible) or to a delayed conformational change to an open-channel conformation after GABA binding. In either case, the delay would not necessarily be related to the GABA concentration. Our observations do not provide an explanation for this delay but we have found that channels activated directly by pentobarbital in similar outside-out patches have a much shorter latency (*unpublished results*). Furthermore, diazepam appeared to reduce the latency (*see* Fig. 10*C*). Other receptors display delayed changes in conformation after ligand binding. For example, $P2X_7$ (P2Z) receptors initially form low-conductance channels after ATP binding but then high-conductance channels appear after a delay (Surprenant et al., 1996): the phenomenon has not yet been explained (Khakh & Lester, 1999). A delay in channel activity can also be seen in cultured spinal cord neurons after application of GABA (Mathers, 1985) and this may be related to the delayed effect of GABA we report here.

Delayed activation of $GABA_A$ channels in outside-out patches has not been reported previously, presumably because patches that were not rapidly activated by GABA would normally be considered not to contain $GABA_A$ receptors. Whole-cell currents have shown no evidence of delayed activation of $GABA_A$ channels, either because their open probability is too low or because delayed activation of these channels is not seen in whole-cells.

Conductance Increases with GABA Concentration

An unusual feature of the delayed channels was the relationship between GABA concentration and channel conductance. Such a relationship has not been reported for $GABA_A$ channels previously. However, it has been shown that both benzodiazepines and barbiturates can increase the conductance of low-conductance $GABA_A$ channels (Eghbali et al., 1997; Guyon et al., 1999; Eghbali, Gage & Birnir, 2000; Birnir et al., 2000b). Furthermore, a relationship between ligand concentration and channel conductance has been reported in cyclic nucleotide- and glutamate-gated channels (Ruiz and Karpen, 1997; Rosenmund et al.,1998; Smith and Howe, 2000). This effect was explained by proposing that each subunit of these receptors has a ligand-binding site and conductance increases as more binding sites become occupied by ligand. The number of conductance levels observed in this study is greater than the normally accepted number of GABA-binding sites and is also greater than the number of subunits. If there are 2 binding sites for GABA per channel (Rabow et al., 1996), 4 conductance states (1 closed and 3 open) might be associated with the differently liganded receptors. We see more conductance states than that but if there were more than 2 binding sites, each with a different affinity for GABA, and if channel conductance depended on which binding sites were occupied, a wide range of conductances that increased with GABA concentration might be obtained (Gage and Chung, 1994). Another explanation for our

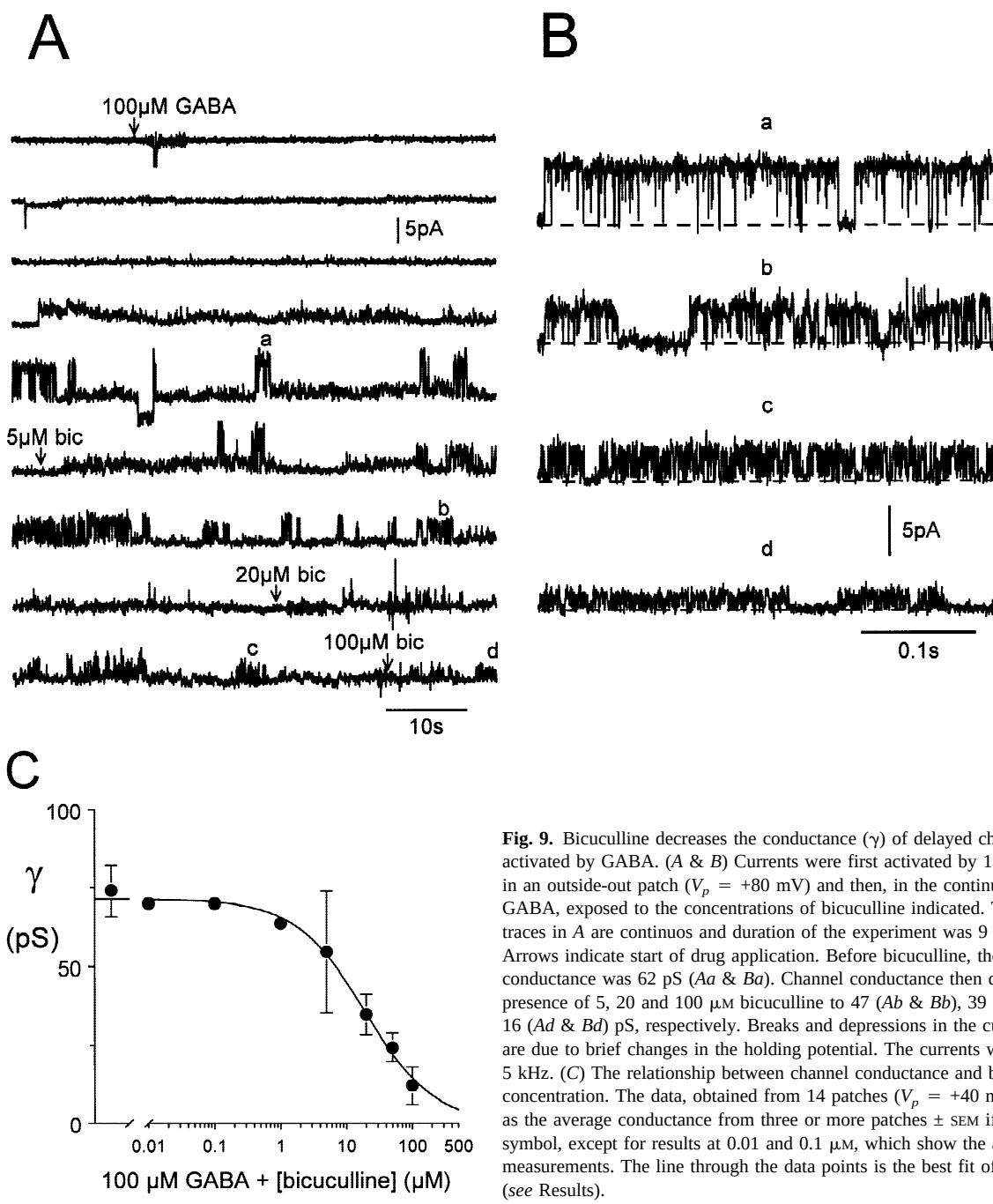

Fig. 9. Bicuculline decreases the conductance (γ) of delayed channels activated by GABA. (*A* & *B*) Currents were first activated by 100 μM GABA in an outside-out patch (V_p = +80 mV) and then, in the continued presence of GABA, exposed to the concentrations of bicuculline indicated. The current traces in *A* are continuos and duration of the experiment was 9 minutes. Arrows indicate start of drug application. Before bicuculline, the maximum conductance was 62 pS (*Aa* & *Ba*). Channel conductance then decreased in the presence of 5, 20 and 100 μM bicuculline to 47 (*Ab* & *Bb*), 39 (*Ac* & *Bc*) and 16 (*Ad* & *Bd*) pS, respectively. Breaks and depressions in the current record are due to brief changes in the holding potential. The currents were filtered at 5 kHz. (*C*) The relationship between channel conductance and bicuculline concentration. The data, obtained from 14 patches (V_p = +40 mV), is shown as the average conductance from three or more patches ± SEM if larger than the symbol, except for results at 0.01 and 0.1 μM, which show the average of 2 measurements. The line through the data points is the best fit of equation 2 (*see* Results).

results that does not require a large number of GABA binding sites is related to Koshland's "induced fit hypothesis" (Koshland, 1958; Yankeelov and Koshland, 1965). In a pentameric receptor, occupation of a binding site by GABA might produce a progression of conformational changes associated with open channels of increasing conductance and terminating in closed (desensitized) conformations. When GABA unbinds, these changes may relax at a rate slow enough to allow the next binding event to drive the channel towards a higher conductance or desensitized state. With appropriate choice of relaxation time constants and binding rates, this model could obviously give a relationship between channel conductance and GABA concentration. This model is in some ways similar to a model put forward by Jones et al. (1998) based on kinetic analysis of rapidly activating $GABA_A$ receptors in outside-out patches. Both models predict that "moulding" of the receptor around the ligand drives the conformational changes that lead to gating but, in addition, the induced fit hypothesis predicts that the

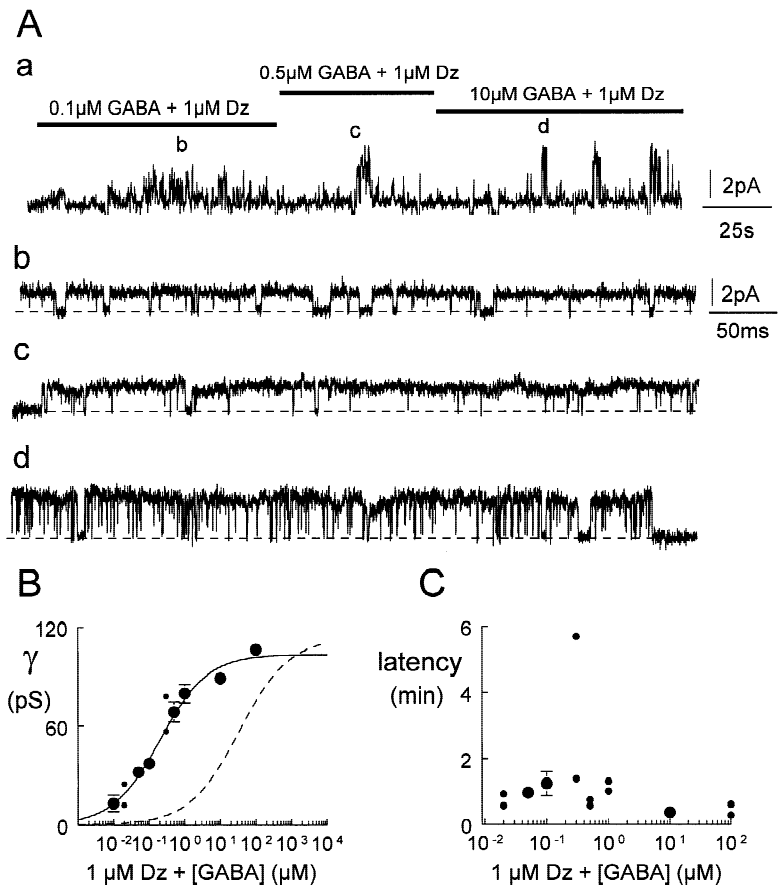

Fig. 10. Effect of 1 μM diazepam on GABA-activated channels. (*A*) In the presence of 1 μM diazepam maximum channel conductance still varies with the GABA concentration. The top compressed trace (*Aa*, 3.8 min) shows an experiment where GABA concentration is changed in the presence of 1 μM diazepam. In the presence of 0.1, 0.5 and 10 μM GABA and 1 μM diazepam the conductance was 39 (*Ab*), 50 (*Ac*) and 85 pS (*Ad*), respectively. Horizontal bars denote the drug applications and breaks in the current record are due to brief changes in the holding potential. The currents were filtered at 5 kHz. The calibrations to the right of trace *Ab* apply to traces *Ab–Ad.* (*B*) Diazepam shifts the relationship between channel conductance and GABA concentration. 1 μM diazepam shifts the GABA activation curve to the left, to lower GABA concentrations. The data, obtained from 15 patches (V_p = +40 mV), is shown as the average conductance from three or more patches ± SEM if larger than the symbol. The smaller symbols are values from individual experiments. The line through the data points is the best fit of equation 1 (*see* Results). The broken line shows the relationship in the absence of diazepam (Fig. 7*A*) for comparison. (*C*) Latency is decreased in the presence of 1 μM diazepam. The delay (latency) between GABA application in the presence of 1 μM diazepam and first appearance of maximum-conductance channels is plotted against GABA concentration. Results shown were obtained from 10 outside-out patches and the larger circles represent the average ± SEM of three or more measurements, whereas the smaller circles represent individual experiments.

progressive "moulding" also affects the ion conduction pathway. The affinity of binding sites for GABA would be expected to increase as these conformational changes proceed (Koshland, 1958; Williams and Morrison, 1979). The observation that the affinity of ligands is higher for desensitized than normal, nonactivated receptors (Jones and Westbrook, 1995) is consistent with this model.

The GABA concentration that gave the half-maximal single-channel conductance (EC_{50}) was 33 ± 6 μM and the EC_{50} for the open probability of the channels was 15 ± 9 μM. The EC_{50} for the mean current was 128 ± 68 μM. These values are within a wide range of GABA EC_{50} values (1 to 500 μM) reported for $GABA_A$ receptors (Sigel et al., 1990; Rabow et al., 1996).

Bicuculline Decreases Channel Conductance

The decrease in the conductance of GABA-activated channels caused by bicuculline is a novel observation. If channel conductance depends on the probability of GABA being bound to a receptor, the opposite effects of GABA and bicuculline concentration on channel conductance are consistent with the idea that they compete for the same binding site.

The bicuculline sensitivity of these receptors (IC_{50} = 19 μM) is lower than for some synaptic $GABA_A$ receptors that can be completely blocked by 10 μM bicuculline although the GABA concentration in the synaptic cleft would be above 1 mM (Edwards et al., 1990). On the other hand, maximal displacement of GABA in rat brain synaptosomes required 100 μM bicuculline (Zukin, Young & Snyder, 1974) and in hippocampal granule cells, slowly-activating and slowly-decaying currents activated by GABA in both immature and mature cells were not fully inhibited by concentrations of bicuculline below 100 μM (Liu et al., 1998). In central amygdala neurons, the bicuculline IC_{50} of $GABA_A$ receptors was similar to that we measured in the outside-out patches (Delaney & Sah, 1999). These differences may be related to the fact that different subunits form the receptors (Rabow et al., 1996).

Diazepam Increases Channel Conductance

Diazepam increased the conductance of the delayed $GABA_A$ channels. The shift of the GABA EC_{50} to a lower value but lack of significant change in the maximum conductance or the Hill coefficient in the presence

of diazepam is consistent with the idea that diazepam increases channel conductance by increasing the affinity of a binding site/s for GABA on the delayed channels (for review *see* MacDonald and Olsen, 1994). However, it may not be the sole explanation since in CA1 pyramidal neurons, diazepam increased the open-probability of spontaneously opening channels although no GABA was present (Birnir et al., 2000a & b) and in this study, it seemed to decrease the latency of the GABA-activated channels. A GABA EC_{50} of 0.2 μM in the presence of diazepam may have pharmacological implications since it is similar to the GABA concentration in the extracellular fluid in the hippocampus (Tossman, Jonsson & Ungerstedt, 1986).

Influence of Patch Configuration

The silent outside-out patches displayed some properties different from those of channels in cell-attached patches on intact neurons (*see* Fig. 3*B*). We have reported previously that delayed channels activated by low concentrations of GABA in cell-attached patches on cultured hippocampal neurons or dentate granule cells and pyramidal CA1 neurons in slices can have a high conductance (Birnir et al., 1994, 2000a; Eghbali et al., 1997). In contrast, delayed channels in outside-out patches activated by low concentrations of GABA had a low conductance, not a high conductance. High-conductance channels could, however, be induced by high GABA concentrations in the silent outside-out patches. Considering how the outside-out patch is formed and that channel complexes must be dragged into the patch, changes in channel characteristics would hardly be surprising, e.g., there could well be differences in phosphorylation of the receptor, protein clustering or interactions with the cytoskeleton (Rabow et al., 1996; Essrich et al., 1998; Wang et al., 1999). In this context, it has been shown that both intracellular and extracellular agents can influence channel conductance (Ali, Catarsi & Drapeau, 1998; Meir & Dolphin, 1998; Derkach, Barria & Soderling, 1999). Whatever the reason for the differences observed, the silent outside-out patches provide an opportunity to study the relationship between ligand concentration and channel conductance and how this intriguing and potentially important relationship may be modulated.

Physiological Relevance

Currents activated during whole-cell recordings from cultured neurons are similar to currents activated in patches containing spontaneous channels. For both configurations application of a high GABA concentration elicited a rapid current response that was not observed in the silent outside-out patches. During whole-cell recordings, however, delayed activation of a new population of receptors was not observed. As we reported previously (Birnir et al., 2000b) the conductance of the spontaneously active channels appears to increase with time and they are modulated by drugs in a similar way to the channels described here that were activated only after a delay. Whether the two kinds of behaviour represent two different molecular entities or two functional states of a single kind of $GABA_A$ receptor remains to be determined.

Sub-micromolar GABA is present extracellularly in the brain but its role in neuronal function is not clear (Tossman et al., 1986; Vautrin et al., 2000). In the presence of low GABA concentrations in cell-attached patches on intact hippocampal neurons in slices (Birnir et al., 1994, 2000a) and on cultured hippocampal neurons (*see* Fig. 3*B*), high conductance channels developed over minutes similar to the delayed activation of channels we have described for the silent outside-out patches. It is conceivable that the low extracellular GABA concentration serves to maintain the $GABA_A$ receptors in intact neurons in their optimal conducting state albeit at a low open probability (Birnir et al., 1994). In the presence of high concentrations of GABA, the open probability would be greatly increased and the channels would respond rapidly.

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