British Journal of Pharmacology (2000) 130, 249–258
www.nature.com/bjp

Activation and block of recombinant $GABA_A$ receptors by pentobarbitone: a single-channel study

*,[1]Gustav Akk & [1]Joe Henry Steinbach

[1]Department of Anesthesiology, Washington University School of Medicine, St Louis, Missouri, MO 63110, U.S.A.

1 Recombinant $GABA_A$ receptors ($\alpha1\beta2\gamma2L$) were transiently expressed in HEK 293 cells. We have investigated activation and block of these receptors by pentobarbitone (PB) using cell-attached single-channel patch clamp.

2 Clusters of single-channel activity elicited by 500 μM PB were analysed to estimate rate constants for agonist binding and channel gating. The minimal model able to describe the kinetic data involved two sequential binding steps, followed by channel opening. The estimated channel opening rate constant is $\sim$1500 s^{-1}, and the estimated equilibrium dissociation constants for the binding steps involved in activation are $\sim$2 mM.

3 Our results show a dose-dependent block of receptors at millimolar concentrations of PB that results in reduced open interval durations. The reduction in mean open time is linearly proportional to PB concentration, indicating that block can be produced by binding of a single PB molecule.

4 Addition of millimolar concentrations of PB in the presence of GABA also produces a reduction of open channel lifetime in addition to a progressive increase in the closed interval durations within a cluster. The data suggest that the receptor contains two or more blocking sites while occupancy of only one of the sites is sufficient for channel block.

5 Neither the blocking rate constant nor return rate from the blocked state(s) is affected by pH (ionization status of the PB molecule) demonstrating that both neutral and anionic forms of PB cause channel block.

British Journal of Pharmacology (2000) **130,** 249–258

Keywords: $GABA_A$ receptors; pentobarbitone; kinetic analysis; single channel

Abbreviation: PB, pentobarbitone sodium salt

Introduction

The $GABA_A$ receptor channel (GABA receptor) is a pentameric protein consisting of one or more types of subunits (Olsen & Tobin, 1990). The protein complex contains binding sites for several clinically important agents such as benzodiazepines, barbiturates, neurosteroids and ethanol, all of which have been shown to modify the receptor function (Olsen & Tobin, 1990; Upton & Blackburn, 1997). Under physiological conditions, the receptors are activated by GABA, but several other compounds such as neurosteroids and barbiturates can also gate the channel (Nicoll, 1975; Cottrell *et al*., 1987).

Electrophysiological experiments have shown that, compared to GABA, the midpoint of the dose-response curve for pentobarbitone (PB) is shifted towards higher agonist concentrations. Depending on the subunit composition of the receptor, the maximum response is reduced, similar or higher, compared to the maximal response to GABA, demonstrating differences in both affinity and efficacy (Akaike *et al*., 1987; Rho *et al*., 1996; Thompson *et al*., 1996; Pistis *et al*., 1997). Single-channel studies on GABA receptors have shown that currents evoked by PB are indistinguishable in conductance from those evoked by GABA, suggesting similarities in the open channel structure for receptors activated by GABA and PB (Mathers & Barker, 1980; Jackson, *et al*., 1982; Rho *et al*., 1996). The mean open dwell times in the presence of PB have been reported to resemble openings activated by GABA (Rho *et al*., 1996), or to differ several-fold (Jackson *et al*., 1982).

*Author for correspondence at: Campus Box 8054, Anesthesiology, 660 South Euclid Ave, Washington University School of Medicine, St Louis, Missouri, MO 63110, U.S.A.
E-mail: akk@morpheus.wustl.edu

PB-mediated agonism is found with a variety of GABA receptor subunit combinations (for review see Lambert *et al*., 1997). In contrast to binding of another class of GABA receptor modulators, benzodiazepines, barbiturates do not require the presence of a γ-subunit (Levitan *et al*., 1988). Further, barbiturates directly gate $\beta2$ and $\beta3$-containing homomeric receptors that are insensitive to GABA (Cestari *et al*., 1996; Davies *et al*., 1997; Wooltorton *et al*., 1997). The binding site for PB differs from that for GABA (Amin & Weiss, 1993). Based on results from mutagenesis experiments it has been proposed that the PB binding site is located within the transmembrane regions of the receptor (Amin, 1999; R. Serafini, personal communication). However, such conclusions are complicated by the findings that the action of many anaesthetics (e.g. enflurane, etomidate) depends on the same residues located in the second and third transmembrane regions of the GABA receptor (for review see Belelli *et al*., 1999).

Barbiturates at concentrations exceeding millimolar values block GABA receptors, leading to a reduced peak response (Akaike *et al*., 1987; Rho *et al*., 1996). PB-elicited block has also been demonstrated in other ion channels such as muscle nicotinic ACh receptors (Gage & McKinnon, 1985; Dilger *et al*., 1997), AMPA type glutamate receptors (Taverna *et al*., 1994; Yamakura *et al*., 1995) and voltage-activated Ca^{2+} channels (ffrench-Mullen *et al*., 1993). Such a wide range of targets coupled with the relatively high concentrations of PB required for block suggests that block does not include binding to a highly specialized high-affinity site but, instead, is rather universal and perhaps involves a conserved site present on many ion channels.

Robertson (1989) found that the potency of PB to gate GABA receptors is pH-dependent. In mammalian dorsal root ganglion neurons, the mean response to 1 mM pentobarbitone was 30 fold smaller at pH 9.4 than at pH 7.4. Since channel block by pentobarbitone was observed even at pH 5.4, when over 99% of pentobarbitone is uncharged, it was concluded that both agonism and block by pentobarbitone are associated with the neutral form of the drug.

In the present work, we have examined agonism and block of recombinant GABA receptors by PB. We report the activation and blocking rate constants for PB, and show that both uncharged and anionic forms of PB can block the receptor.

Methods

Expression systems

Rat GABA receptor cDNA was generously provided by Drs A. Tobin (University of California, Los Angeles, U.S.A.; α1, β2) and D. Weiss (University of Alabama, Birmingham, U.S.A.; γ2L) and subcloned into a CMV promoter-based expression vector pcDNAIII (Invitrogen Corp., San Diego, CA, U.S.A.). The GABA receptors were expressed in human embryonic kidney (HEK) 293 cells using transient transfection based on calcium phosphate precipitation (Ausubel *et al*., 1992). A total of 7.5 μg of DNA per 35 mm culture dish in the ratio of 2 : 2 : 1 (α:β:γ) was used. The medium was changed 12 h later, electrophysiological experiments commenced $\sim$36 h after the start of transfection.

Electrophysiology

Electrophysiological experiments were performed using the patch clamp technique in the cell-attached configuration (Hamill *et al*., 1981). The bath solution contained (in mM): NaCl 140, KCl 5, $MgCl_2$ 1, $CaCl_2$ 2, glucose 10 and HEPES 10; pH 7.4. The pipette solution contained (in mM): NaCl 120, KCl 5, $MgCl_2$ 10, $CaCl_2$ 0.1, tetraethylammonium 20, 4-aminopyridine 5, glucose 10, HEPES 10: pH 7.4. In addition, the pipette solution contained the indicated concentrations of PB sodium salt and GABA. In some experiments the pH of the pipette solution was adjusted to 9.1. The interior of the pipette was held at +60 mV unless indicated otherwise. We assume that the cell-membrane potential was ~ -40 mV, thus the total potential difference across the patch was -100 mV. The unitary conductance was determined from the slope of the I-V plot which was linear at pipette potentials between -60 and 60 mV. Experiments were performed at 22°C. Single-channel currents were recorded with an Axopatch 200B amplifier, low-pass filtered at 10 kHz, acquired with a Digidata 1200 Series Interface at 50 kHz using pClamp 7 software (Axon Instruments, Foster City, CA, U.S.A.) and stored on a PC hard drive for further analysis.

Analysis of gating kinetics

For the determination of open interval durations in the presence of PB, a patch-clamp record was broken into 10 s segments (program PSNIP, Dr C. Lingle, Washington University, St. Louis, MO, U.S.A) for easier handling. Program PRE (QUB Suite, Drs A. Auerbach, F. Qin and F. Sachs, SUNY at Buffalo, Buffalo, NY, U.S.A.) was used to isolate stretches of homogenous data, omitting periods of prolonged inactivity, noise or overlapping currents. Files that contained persistent overlapping activity were not analysed. For event detection, the data was low-pass filtered at 2–4 kHz and idealized using the segmented-k-means algorithm (program SKM, Qin *et al*., 1996; 1997). Mean open and closed interval durations were estimated from the histograms of interval dwell times. Currents from a single patch typically contained 400–2000 events. In the present experiments the open time distribution was well described by a single exponential when PB alone was used.

The mean open interval duration *vs* PB concentration curves were fitted using the following equation:

$$\tau_{on} = 1/(\alpha + k_{+B}[PB]) \quad (1)$$

where τ_{on} is the mean open interval duration, α is the channel closing rate constant, k_{+B} is the channel blocking rate constant, and [PB] is PB concentration. This equation describes an open channel blocking mechanism; according to the equation, an opening can be terminated by one of two processes: channel closing or channel block. At low [PB] termination of an opening is dominated by α (channel closing), while as [PB] is raised the relative weight of k_{+B} [PB] increases.

Clusters of channel openings from individual receptors were elicited by 500 μM PB. Clusters were defined as series of openings separated by closed intervals shorter than a critical duration (τ_{crit}). In the present experiments, most clusters were separated from each other by prolonged periods of inactivity lasting several seconds. We assume that during the intercluster silent periods, the receptors in the patch are desensitized (*cf*. Sakmann *et al*., 1980), however, due to their extremely long durations, and dependence on the number of channels in the patch, it is not possible to investigate the rate of recovery from desensitization using the cell-attached patch-clamp technique. We chose τ_{crit} to be 500 ms, which is $\sim$10 times longer than the longest closed time constant within clusters, and $\sim$10 times shorter than the intervals between clusters. Thus, according to Colquhoun & Sigworth (1995), the percentage of misclassified intracluster events is $\sim$0.005% and of intercluster events is $\sim$9.5%. The isolated clusters were low-pass filtered at 2.5 kHz and idealized using program SKM. The single-channel clusters consist of dwells in all states of Scheme 1 (see below). Rate constants were estimated from the interval dwell times using program MIL (dead time was 70 μs) and various kinetic schemes (Figure 4A–E).

When GABA was co-applied with PB, a variable τ_{crit} was used to identify clusters. The value for τ_{crit} depended on the dwell times within a cluster but was never less than 10 times the slowest closed interval component within a cluster. For example, to separate clusters elicited by 50 μM GABA and 200 μM PB, we used τ_{crit} of 150 ms while the slowest component of the intracluster closed interval duration histogram was 4.8 ms. On the other hand, in the presence of 50 μM GABA and 2 mM PB, the slowest intracluster closed dwell time constant was 41 ms, and τ_{crit} was 500 ms. The intercluster intervals were sufficiently long, as our calculations showed that the typical theoretical misclassification of intercluster events was $<$10%. The records were idealized (f_c = 2–3 kHz) using program SKM and mean open and closed interval durations obtained from the respective histograms. Histograms shown in Figure 7 contained 16,000–26,000 open channel events. The analysis was confined to patches which contained few or no overlapping clusters, the presence of which can artificially reduce the mean cluster duration. This happens because longer than average clusters are more likely to be overlapped by a neighbouring cluster in a patch which contains many channels. Since overlapping clusters are discarded from analysis, the record would contain a smaller number of longer than average clusters leading to a reduction in the mean cluster duration.

Drugs

All chemicals including PB sodium salt and GABA were obtained from Sigma (St Louis, MO, U.S.A.).

Results

Single-channel conductance and mean open time for receptors activated by PB

Typical single-channel currents elicited by 200 μM PB are shown in Figure 1A. The openings in this patch have an average duration of 1.9 ms, and the activity takes place as isolated openings with no clusters evident. The openings are homogeneous in regard to their amplitude and have a single channel conductance of 26 pS. The single-channel conductance varied in different patches, in six patches it ranged from 19 to 26 pS (mean $\pm$ s.d.mean 20.5 $\pm$ 3.6 pS).

As the PB concentration is increased, the openings become shorter. In the presence of 10 mM PB, the average open duration (τ_{on}) is only 0.16 ms (Figure 1B). At all PB concentrations, the open time distributions are described by a single exponential.

Figure 2A shows the relationship between the mean open duration and the concentration of PB. There is a gradual decrease in the τ_{on} at higher PB concentrations. Using Equation 1 (see Methods) to fit the relationship yields the channel closing rate constant (α), 300 s^{-1}, and the channel blocking rate constant (k_{+B}), 0.56 μM^{-1} s^{-1}. The results are also shown in Table 1. Equation 1 describes a blocking mechanism in which the forward rate for block increases linearly with PB concentration, consistent with the idea that binding of a single PB molecule can produce block. At low PB concentrations, the openings are terminated mainly by 'normal' channel closing and approach a maximal open duration of $\sim$3 ms, while at higher PB concentrations channel block becomes predominant.

We will describe the concentration-dependent reduction in the mean open duration of GABA receptor currents by PB as 'block', and refer to a component in the closed duration distribution as resulting from a dwell in the blocked state. However, we do not mean to imply any particular molecular model for this process (e.g. channel occlusion).

Figure 2B shows that the mean open time is not influenced by changes in the membrane potential. The data at each PB concentration comes from a single patch. The single-channel

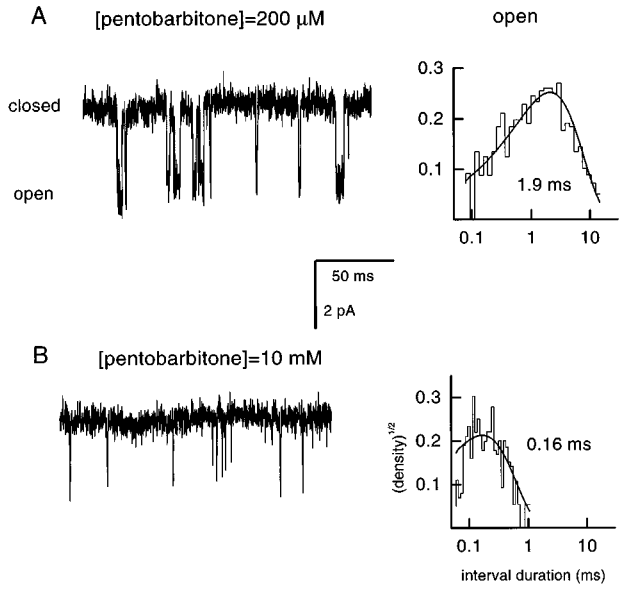

Figure 1 Sample currents recorded from cell-attached patches on HEK cells transiently transfected with $\alpha1\beta2\gamma2$ subunits, and open interval duration histograms. The average open interval duration in the presence of 200 μM PB is 1.9 ms (A). When PB concentration is increased to 10 mM, the open interval duration is reduced to 0.16 ms (B). Outward chloride flux is shown downward. Each histogram is from one patch, 735 events in (A), 407 events in (B). For determination of open time, currents were filtered at 2 kHz (A) or 4 kHz (B).

Table 1 The channel closing and blocking rate constants

Receptor, conditions	k_{+B} ($\mu M^{-1}s^{-1}$)	α (s^{-1})
$\alpha1\beta2\gamma2$, PB	0.56 $\pm$ 0.14	300 $\pm$ 25
$\alpha1\beta2\gamma2$, GABA + PB	0.32 $\pm$ 0.09	189 $\pm$ 24

The channel closing rate constant and blocking rate constant for the $\alpha1\beta2\gamma2$ receptor in the presence of PB or 50 μM GABA + PB. The values are best fit parameter values $\pm$ standard deviation estimated from the fit. Curve-fitting was performed using Equation 1. The data is from Figures 2 and 7A.

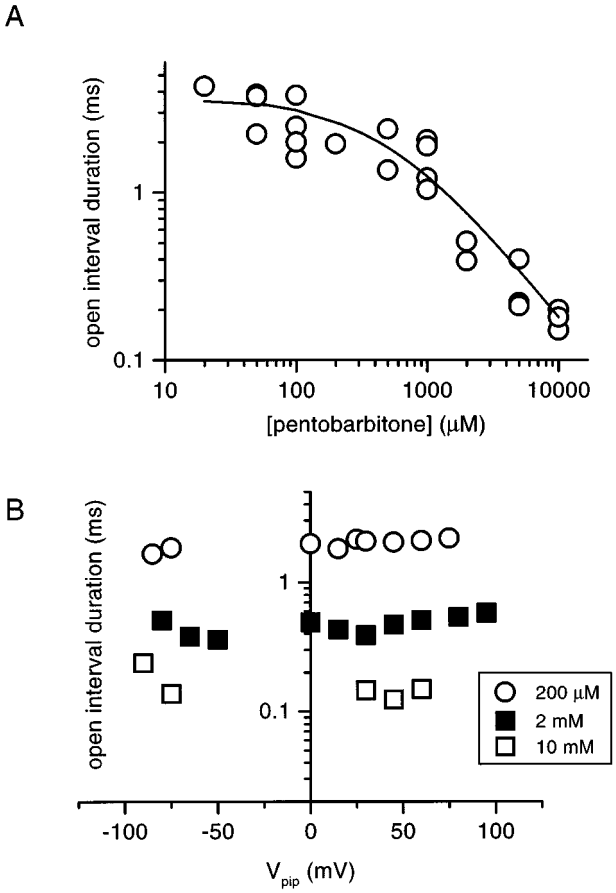

Figure 2 The mean open interval duration of $\alpha1\beta2\gamma2$ receptors is reduced at high PB concentrations (A). Each data point represents data from one patch. The channel open durations were obtained after filtering the data at 2–4 kHz. The data recorded in the presence of PB concentrations over 1 mM were filtered at 4 kHz. The curve was fitted using Equation 1. The results of the analysis are shown in Table 1. The mean open interval duration is not affected by voltage (B). Data for each PB concentration come from a separate patch. Data for different V_{pip} at each concentration come from the same patch. The regression coefficient demonstrated voltage-sensitivity of $>$1000 mV per e-fold change.

open interval durations were measured at different PB concentrations (200 μM, 2 mM, 10 mM) in order to separate the effect of voltage on channel closing and channel block. At 200 μM PB, the termination of a channel opening is dominated by closure of the channel (3 : 1 ratio, α:k_{+B}[PB]). At 2 and 10 mM PB, the channels are preferentially blocked by PB (1 : 3, 1 : 17 ratio, α:k_{+B}[PB]). Thus, if the closure of the activation gate were voltage-dependent, the mean open time at 200 μM PB would exhibit voltage-dependency. On the other hand, if voltage affected channel block by PB, then the mean open time in the presence of 2 – 10 mM PB would be scaled by membrane potential. Our results show that neither of these processes is affected by voltage.

Analysis of the kinetics of activation by pentobarbitone

In the presence of 500 μM PB, the single-channel activity takes place in easily recognizable clusters. At lower or higher concentrations, clusters were difficult to identify due to prolonged closed interval durations and the relatively high density of receptors in the patch. To gain some insight into the mechanism by which PB activates GABA receptors, we analysed the activity in clusters of single-channel openings. In this analysis, it is assumed that an individual cluster originates from the activation of a single ion channel, thereby making it possible to compute activation rate constants for PB-activated GABA receptors. Different clusters are likely to result from different receptors, which recover from desensitized states. We have assumed that the activity of each receptor (each cluster) reflects the activation properties of the population of receptors, and have combined the data from all clusters in estimating the activation parameters.

Typical clusters recorded in the presence of 500 μM PB are shown in Figure 3. The open probability (P_o) of the receptor, defined as a fraction of time the receptor spends in the open state (within a cluster), is $\sim$0.05. The open and closed interval histograms are shown in Figure 4.

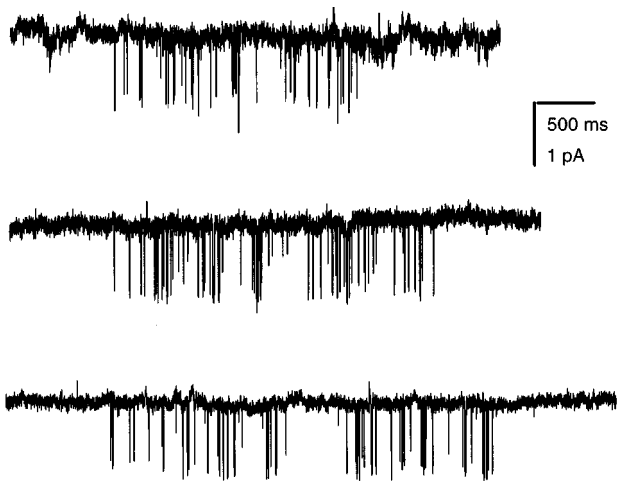

Figure 3 Sample clusters of $\alpha 1\beta 2\gamma 2$ receptors elicited by 500 μM PB. The clusters were defined as series of openings separated by closed intervals shorter than 500 ms (see Methods). Outward chloride flux is shown downward.

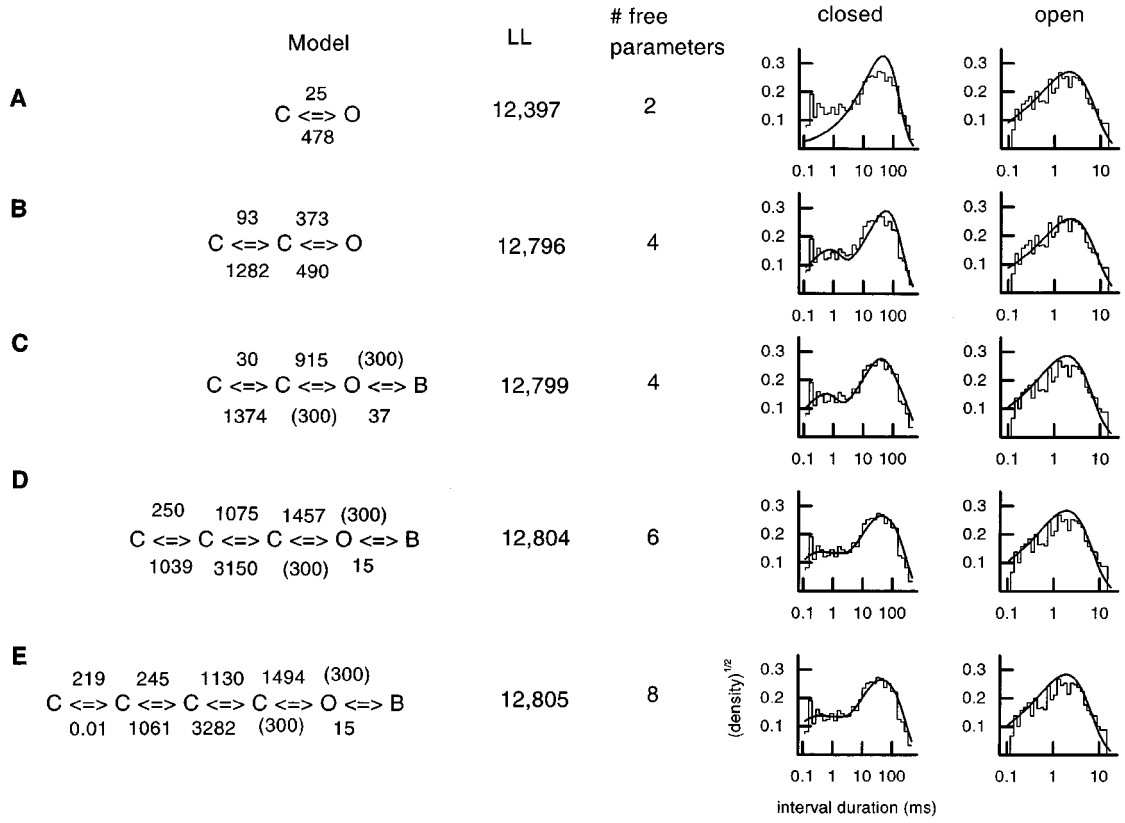

Figure 4 Kinetic analysis of single-channel data obtained at 500 μM PB. The analysis was performed on data from three patches containing in total 3373 openings. The currents were analysed using program MIL and different kinetic schemes. In each model: C, closed receptor, O, open receptor, B, blocked receptor; rate constants for left to right transitions are shown above the transition symbol. Rate constants that were constrained are shown in parentheses (C – E). All rates are given in units of s^{-1}. The goodness of the fit is shown as a log-likelihood next to each model (LL). The closed and open time histograms also contain a solid line that shows the interval distribution predicted by the respective model and rate constants. Data was filtered at 2.5 kHz, dead time was 0.07 ms.

To estimate values for rate constants underlying activity we used a kinetic analysis program MIL (see Methods). The data set consisted of recordings from three patches containing 25 clusters with 3373 channel openings. The dead time was set at 0.07 ms. We started with a simple two-state, closed ⇆ open model, gradually adding states (see Figure 4), to determine the simplest model we could use. The addition of kinetic states is justified if the difference of log-likelihoods corresponding to the compared models is above a certain value which depends on the number of added free parameters and a *P* value desired (Horn, 1987). With some models (Figure 4C–E) we used two constraints in order to reduce the number of free parameters. The channel closing rate constant (α) and the blocking rate constant (k_{+B}) were constrained to values obtained from the analysis of the blocking dose-response curve ($\alpha = 300\ s^{-1}$, $k_{+B} = 0.6\ \mu M^{-1}\ s^{-1}$). As shown in Figure 4, the addition of two free parameters between models A and B resulted in an increase in the log-likelihood (LL) by 399. For an assumed probability of 0.05 that the increased log-likelihood would arise by chance, the difference in LL for each added state should be greater than 3. Clearly, model B is superior to model A. The results shown in Figure 4 demonstrate that model D is significantly better than C, B or A, while model E is not an improvement over D. Thus, our results suggest that a model consisting of three closed states, one open state and one blocked state is the minimal kinetic scheme that adequately describes single-channel currents in the presence of 500 μM PB:

$$\begin{array}{ccccccccc} & [PB]\,k_{+1} & & [PB]\,k_{+2} & & \beta & & [PB]\,k_{+B} & \\ C & \leftrightarrow & AC & \leftrightarrow & A_2C & \leftrightarrow & A_2O & \leftrightarrow & A_3B \\ & k_{-1} & & k_{-2} & & \alpha & & k_{-B} & \text{(Scheme 1)} \end{array}$$

where k_{+1}, k_{+2}, k_{-1}, k_{-2} refer to the agonist association and dissociation rate constants, β is the channel opening rate constant, α is the channel closing rate constant, and k_{+B} and k_{-B} are channel blocking and unblocking rate constants.

Thus, a resting receptor has to bind two PB molecules in order to open. According to our calculations, the receptor has a rather low affinity for PB. The two binding sites have similar affinities, $K_{d1} = 2078\ \mu M$, and $K_{d2} = 1465\ \mu M$. The fully-liganded receptor opens with an opening rate constant of $\sim 1500\ s^{-1}$ and the calculated efficacy of PB at activating the receptor (β/α) is 4.9.

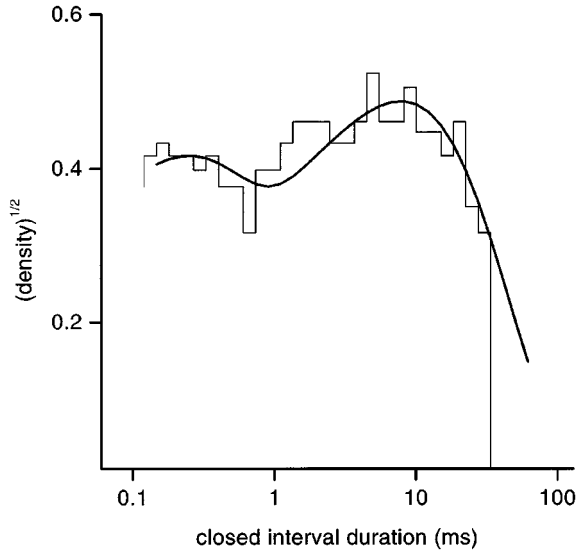

Figure 5 The closed interval duration distribution in the presence of 100 μM PB. The mean duration of the briefer component is $1/(\beta + k_{-2})$ and the relative area is $\beta/(\beta + k_{-2})$. For the slower component, the relative area is 1-(β/(β-k_{-2})), and the mean duration is the best fit. The solid line is calculated using the gating rate constants shown in Figure 4D. Histogram corresponds to one patch with 424 events.

As an independent approach to estimating the rate constants β, α and k_{-2} for Scheme 1, we analysed the behaviour of bursts of single-channel activity elicited by 100 μM PB. Under such conditions the single channel activity takes place as individual bursts (no clusters can be seen), and block is insignificant (see Figure 2A). The closed interval duration histogram of single-channel activity recorded at 100 μM PB is shown in Figure 5. The bursts at low agonist concentrations contain information about the open state and the immediately adjacent closed state. In terms of Scheme 1, these are A_2C ⇆ A_2O (neglecting the blocked state). Accordingly, we used a simplified, 3-state model C ⇆ C ⇆ O. Our analysis of data from one patch (424 events) yields: $\beta = 1613\ s^{-1}$, $\alpha = 322\ s^{-1}$ and $k_{-2} = 3328\ s^{-1}$. These estimates agree well with the activation rate constants obtained from kinetic analysis of clusters recorded in the presence of 500 μM PB (Figure 4D). Also, the solid line in Figure 5 is calculated using the gating rate constants shown in Figure 4D. The mean duration of the briefer component is calculated from $1/(\beta + k_{-2})$ (0.22 ms). The relative areas of the briefer and slower components are calculated from the ratio of the channel opening rate constant over the sum of the opening rate constant and the agonist dissociation rate constant ($\beta/(\beta + k_{-2})$, 0.32 : 0.68). The mean duration of the slower component is arbitrary and, in this patch, equals 6.4 ms. This time constant bears no relevance to the activation rate constants since it is likely the result of dwells in several closed states of many channels in the patch.

Properties of the blocked state(s)

Both the single-channel clusters obtained at 500 μM PB and the relationship between mean open duration and [PB] can be well explained by the assumption that the receptor has only one site at which binding of PB results in block. However, there is some evidence contradicting this. With only one blocking site per receptor, the blocked state would be detected as a distinct component in the closed interval duration histogram. Our analysis at 500 μM PB gives an estimate of 67 ms for the residence in the blocked state. As PB concentration is increased, the relative weight of the component would increase. Its time constant, however, would remain unaffected. At high PB concentrations (mM) the channel activity should take place as short openings separated from each other by gaps of $\sim$70 ms. In our experiments we could not detect the presence of such a component at PB concentrations above 500 μM. Further, we noticed that at high PB concentrations no clusters could be detected and the closed time histograms could be described by a single exponential with a time constant longer than the dwell time corresponding to the blocked state within clusters recorded with 500 μM PB. For example, at 5 mM PB the mean closed time duration was 154 ms (one patch). We suspect that the mean lifetime of the blocked state at 5 mM PB is even longer but is reduced to this value due to the high number of receptors in the patch. This observation shows that at higher [PB] the receptor remains longer in the blocked state, suggesting that the receptor contains two or more blocking sites. If the receptor contains several blocking sites that can be occupied in sequence, then, both the blocking and unblocking rate constants (shown in Figure 4) are in fact composite rates reflecting the affinity of all blocking sites. For example, if the receptor contains two

equivalent blocking sites, then each of the microscopic blocking rate constants equals one half of the composite blocking rate constant k_{+B}. The relationship between the apparent (composite) unblocking rate constant, k_{-B} and microscopic blocker dissociation rate constant is less trivial. In addition to being dependent on the number of blocking sites, the apparent unblocking rate constant is affected by re-binding of blocker molecules.

Effects of high concentrations of PB on GABA-activated single-channel currents

We also studied single-channel properties of the GABA receptor when GABA is co-applied with PB. Sample clusters along with open and closed time histograms are shown in Figure 6A,B. The clusters consist of homogenous openings with a single-channel conductance of 19 pS. The durations of the longest component in the distributions of open and closed intervals within a cluster measured in the presence of 50 μM GABA and 100–5000 μM PB are shown in Figure 7A, B. The results show that there is a gradual decrease in the open interval durations as PB concentration is increased, similar to what is shown above for receptors activated by PB alone. The results of fitting using Equation 1 are summarized in Table 1. In the presence of 50 μM GABA, PB reduces the mean open duration with an apparent blocking rate constant of 0.32 $\mu M^{-1}s^{-1}$. Thus, GABA receptors activated by GABA or PB are blocked by PB with a similar rate.

Figure 7B shows the mean duration of the slowest component in the closed interval durations within a cluster. We believe that this component is related to the blocked state of the receptor because its area is increased at elevated PB concentrations. Since currents within a cluster arise from an individual receptor, these results suggest that at higher PB concentrations the receptor dwells longer in a blocked state(s). These results agree with our hypothesis that the GABA receptor contains two or more blocking sites that can be occupied at the same time, while occupying one or more sites produces block.

A cluster represents activity from a single ion channel, and it is assumed that between clusters all receptors are in a long-lived desensitized (inactive) state (Sakmann *et al*., 1980). Thus, the cluster duration is inversely related to the rate of entry into the long-lived desensitized state. Table 2 shows the mean cluster duration and P_o for clusters activated by GABA + PB. Although the mean cluster durations are highly variable, they show no systematic changes as PB concentration is increased (and P_o reduced). Thus, the desensitization rate remains unchanged at different PB concentrations. High concentrations of PB result in reduced P_o as the result of reduction in time spent in the open state and increase in the time spent in the blocked state. Since there is no relation between the rate of desensitization and P_o, we conclude that the receptors can desensitize from the blocked state. Theoretical cluster durations (τ_T) shown in Table 2 are calculated assuming there is a direct inverse relationship between the cluster duration and P_o.

The effect of pH on PB-mediated block of single-channel currents

A PB molecule can exist in ionized and neutral forms. Most of the experiments here were performed at pH 7.4 at which ~20% of PB is in an anionic form. In order to investigate which form of PB is responsible for channel block, we compared currents recorded at pH 7.4 to those at pH 9.1 (at which over 90% is anionic). Our experiments show that with 5 mM PB (no GABA present) when the predominant way of terminating an opening is

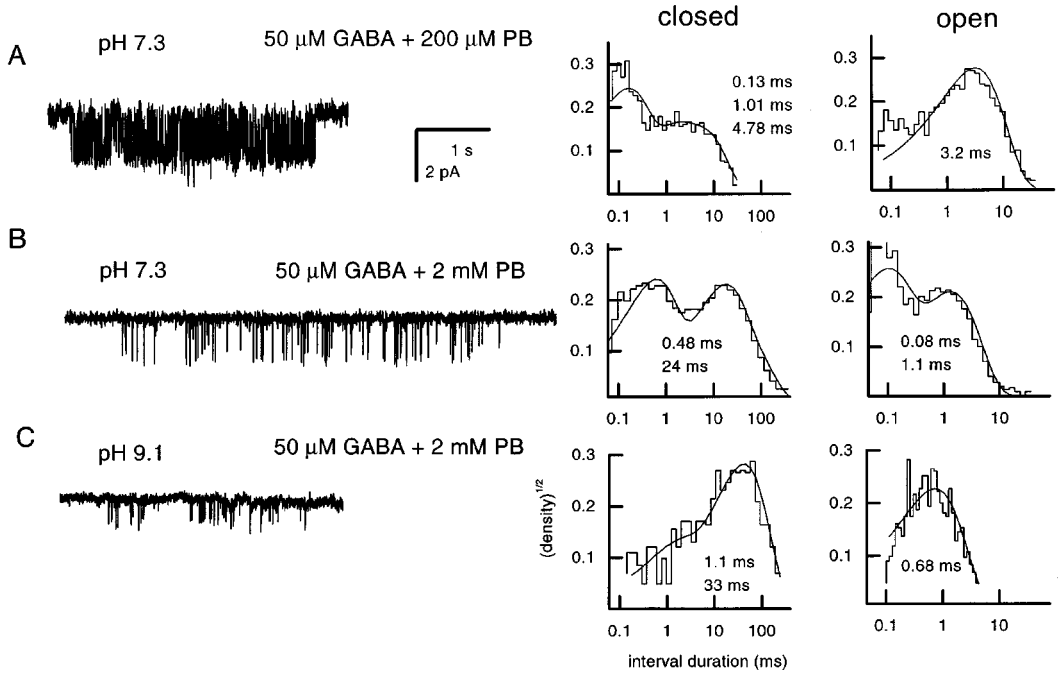

Figure 6 Sample clusters and open and closed interval duration histograms of $\alpha 1\beta 2\gamma 2$ receptors activated by GABA and varying concentrations of PB. The currents were recorded at pH 7.4 (A, B) or 9.1 (C), and activated by 50 μM GABA and 200 μM PB (A) or 50 μM GABA and 2 mM PB (B, C). The time constants for closed and open dwell intervals are shown in histograms. The results show that increasing concentrations of PB result in channel block and cause shorter openings and longer closed interval durations (compare A to B). Elevated pH at the same concentrations of GABA and PB results in shorter openings but has little effect on the slowest component of the closed interval duration histogram (B and C). The histograms correspond to the patch from which a representative cluster is shown.

via channel block ([PB] $k_{+B} >> \alpha$), the average open interval duration is not pH dependent. The average open time at pH 7.4 is 0.28 ms ($n=3$), compared to 0.31 ms at pH 9.1 ($n=3$). It is possible that pH affects the channel closing rate constant, α. Since the apparent open duration depends on both k_{+B} and α (see Equation 1) an increase in one of the variables may be offset by a decrease in another, resulting in no net changes. We, therefore, compared the open durations of channels activated by 100 μM PB (when $\alpha >>$ [PB] k_{+B}) at pH 7.3 and 9.1. Our results show no significant differences, the mean open duration was 2.5 ± 1.0 ms ($n=4$) at pH 7.3, and 2.8 ± 0.7 ms ($n=2$) at pH 9.1. Therefore, k_{+B} does not depend on whether PB is in anionic or neutral form, and block occurs with both forms of PB.

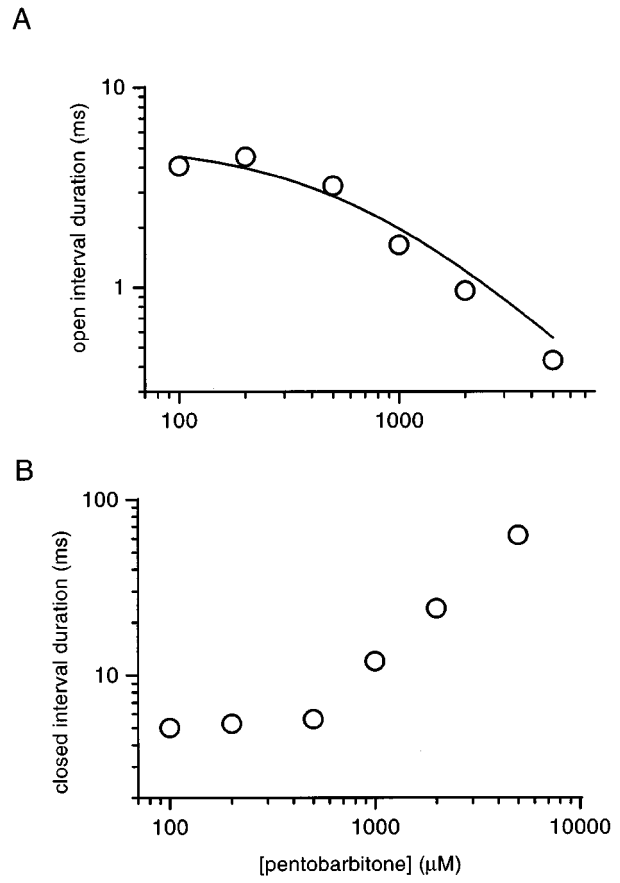

Figure 7 The slowest components of the open and closed interval duration histograms of clusters recorded in the presence of 50 μM GABA and varying PB concentrations at pH 7.4. (A) The mean open duration is reduced at increasing PB concentrations. The curve was fitted using Equation 1. The results are shown in Table 1. (B) The mean duration of the longest component of closed intervals within a cluster is increased at high PB concentrations. We assume that this component is associated with the blocked state (see Results). Each point corresponds to data from one patch.

Figure 2B shows that in the presence of 10 mM PB, when channel closure occurs mainly *via* channel block, the mean open duration does not depend on the membrane potential (see above). In addition, we investigated whether channel block at pH 9.1, when PB exists mostly in charged form, depends on the transmembrane voltage. Our results show that with 5 mM PB, in the range of pipet potentials between +50 and +110 mV, voltage had minimal effect on the channel mean open duration (0.36 ms at +50 mV; 0.43 ms at +110 mV). Thus, we conclude that the channel blocking rate constant is voltage-independent.

We next decided to examine whether the apparent unblocking rate constant, k_{-B}, depends on the state of PB. To do so, we took advantage of a previous finding that when GABA and relatively high concentrations of PB are coapplied there is significant channel block resulting in an increase in the closed interval durations within a cluster. Under such conditions, the slowest component in the closed time histogram that scales with [PB] is associated with the return rate from the blocked state(s). Thus, changes in dwell times measured at different pH can be interpreted as changes in the unblocking rate. We recorded single-channel currents in the presence of 50 μM GABA and 2 mM PB at pH 9.1 (Figure 6C), and compared them to data recorded at pH 7.4 (Figure 6B). Under these conditions a significant amount of block is present that can be detected as prolonged closed intervals within a cluster (compare Figure 6B to A). We found that at pH 9.1, the mean duration of the slowest closed interval component within a cluster was 33 ms. Compared to pH 7.4 ($\tau_{closed} = 24$ ms) this represents a relatively minor effect. Therefore, we conclude that the unblocking rate constant is not affected by pH, and the affinity of PB for the blocking site does not depend on the ionization status of the molecule.

The apparent amplitudes of currents are smaller at pH 9.1 than at pH 7.4 (compare Figure 6B to C). We did not examine whether this was caused by a change in the single-channel conductance or a shift in the reversal potential for ion movement through the channel.

Discussion

The results demonstrate that recombinant $\alpha 1\beta 2\gamma 2$L GABA receptors transiently expressed in HEK cells can be activated by PB. High concentrations (mM) of PB cause channel block leading to termination of an opening. We have found that the single-channel conductance of the $\alpha 1\beta 2\gamma 2$ GABA receptor is similar when the receptors are activated by GABA (G. Akk, unpublished observations), PB or GABA+PB. This has previously been shown for GABA receptors from mouse spinal neurons (Mathers & Barker, 1980; Jackson *et al*., 1982; Macdonald *et al*., 1989) and from rat hippocampal neurons

Table 2 Receptor desensitization is not affected by PB-elicited block

50 μM GABA+	τ_C (ms)	*# Clusters*	P_o	τ_C*P_o (ms)	τ_T (ms)
200 μM PB	1952±1465	44	0.59	1150	1952
500 μM PB	5779±3185	12	0.53	3060	2169
1000 μM PB	2273±2310	41	0.19	430	6053
2000 μM PB	6260±3236	18	0.06	380	16667

The cluster duration (τ_C) and P_o in the presence of 50 μM GABA and varying PB concentrations. The product τ_C*P_o estimates the total open time in a cluster. If cluster termination (desensitization) occurs only from the open state, it should be constant. Theoretical cluster duration (τ_T) is calculated assuming a constant τ_C*P_o value using the value for τ_C obtained in the presence of 50 μM GABA and 200 μM PB. If cluster termination occurred only from the open state, the clusters would have a mean duration of τ_T.

(Rho *et al*., 1996), and suggests that the structure of the open pore is similar when the gate is opened by GABA or PB.

Our kinetic analysis suggests that the minimal kinetic scheme able to account for experimental data consists of three closed states, one open state and one blocked state. Even though the kinetic analysis was performed at only a single pentobarbitone concentration some of the results are confirmed by independent analyses. We have analysed channel openings at 100 μM PB. Here, the channel activity does not take place in clusters, however, it is possible to analyse the bursting behaviour of the channel. By examining the distribution of open and closed interval durations, it is possible to get independent estimates for β, α and k_{-2}. These values agree well with the estimates obtained from analysing single-channel clusters. It has been shown previously that the Hill coefficient of the whole-cell dose-response curve is $\sim$2 (Akaike *et al*., 1987; Rho *et al*., 1996). This agrees with our single-channel results requiring the resting receptor to bind 2 PB molecules before channel opening.

It was shown previously that in the presence of GABA the opening rate constant of GABA receptors from rat cerebellar neurons is 6000 s^{-1} (Maconochie *et al*., 1994). Our results show that the channel opening rate constant is $\sim$1500 s^{-1} (a difference of 0.8 kcal mol^{-1} in free energy) when the receptor is activated by PB. Thus the lower activating potency of PB is mostly due to the lower affinity of the receptor to PB rather than lower efficacy. A decrease in microscopic affinity and channel opening rate, as shown here, would produce a rightward shift in the whole cell dose-response curve and a reduction in peak response. On the other hand, GABA-elicited currents can be potentiated by PB at micromolar concentrations suggesting the presence of an additional, high-affinity PB site on the receptor (Akaike *et al*., 1985; 1990). Therefore the PB binding sites for direct activation and modulation of GABA-activated currents possess different apparent affinities for PB. The two sites may be distinct. However, it is also possible that occupancy of the GABA binding site changes the affinity of a single class of single PB binding site, so the PB site has different affinities in the absence and presence of GABA.

Our studies of block by PB support the following model for block: (i) the binding of a single PB molecule produces block; (ii) block can be produced by either a charged or an uncharged PB molecule and (iii) the forward rate for block is independent of membrane potential. Although a simple open channel block mechanism has been proposed for the actions of PB (Rho *et al*., 1996; Wooltorton *et al*., 1997), our data are not fully consistent with this idea. Our main observation is that the apparent blocked duration increases as the PB concentration is increased, which is not easily accommodated by a simple open channel block at a single blocker binding site (Neher & Steinbach, 1978).

In addition, the results show that in the presence of GABA + PB, the mean lifetime of the blocked state increases as the PB concentration is raised. This also supports our hypothesis that the GABA receptor contains several sites, binding to which of PB leads to channel block. Interestingly, the actual values for the mean duration of the component assigned to the blocked state are different for GABA + PB compared to PB-activated receptors. When the receptors are bathed in 50 μM GABA + 5 mM PB, the mean durations associated with the blocked state reach $\sim$60 ms. With PB acting as both an agonist and a blocker, the mean duration of the blocked state is $\sim$70 ms at only 500 μM PB. Thus, 10 fold more PB is required for the receptor to remain in the blocked state when the receptor is activated by GABA + PB instead of PB alone. Theoretically, if block occurred *via* an independent mechanism the duration of sojourns in the blocked state should not depend on the nature of the agonist. It is possible that when the GABA binding site is occupied the structure of the PB blocking site is altered in a way that results in a higher apparent dissociation rate for PB. Thus, allosterically caused changes in the site which mediates occlusion of the channel may be responsible for differences in the apparent dissociation of PB from the blocking site. However, such changes do not have any notable effect on the single-channel conductance suggesting there is some physical separation between the PB blocking site(s) and the rate limiting activation energy barrier in the diffusion path that determines single-channel conductance.

Previous studies of the block of the muscle-type nicotinic ACh receptor by barbiturates have also found that increased concentrations of barbiturates lead to longer closed dwell times (Gage & McKinnon, 1985). Dilger *et al*. (1997) proposed a sequential blocking model where two barbiturate molecules can bind to the muscle-type nicotinic receptor, to explain an increase in the gap duration within a burst. Our findings can be explained by a similar model for barbiturate block of GABA receptors.

The results of our experiments at different external pH suggest that neither the blocking rate constant, k_{+B}, nor the apparent unblocking rate constant, k_{-B} depend on whether the pentobarbitone molecule is in the neutral or anionic form. The interpretation regarding the blocking rate constant is supported by the observations that the mean open time at low pentobarbitone concentrations is not affected when the pH is increased from 7.4 to 9.1 (indicating no change in the intrinsic channel closing rate), and that the mean open time at high pentobarbitone concentration also is not affected (indicating no change in k_{+B}). The conclusion about the apparent unblocking rate constant, however, is more problematic. Although the data on closed times in the presence of GABA + PB suggest no major change in the component assigned to blocking events, it is possible that changes in extracellular pH result in two opposing processes. First, pH affects the intrinsic unblocking rate constant *via* actions on the receptor. Second, the charged pentobarbitone molecule has a different affinity for its binding site due to the presence of negative charge. If the two processes have effects of similar magnitude on the GABA receptor kinetic properties, then there would be no net effect on the duration the receptor remains blocked. The results do not allow us to distinguish between the two possibilities. The latter interpretation should not be discarded, especially in light of previous results on the effect of extracellular pH on GABA receptor function (Krishek *et al*., 1996). Our data are not in disagreement with results by Robertson (1989) who observed agonism and block by PB at pH values 5.4–9.4. Robertson (1989) concluded that the neutral species of pentobarbitone produced both agonism and block. However, his conclusion about block is complicated by the fact that responses to pentobarbitone were very small or absent at pH 9.4, and he was not able to directly assay block by pentobarbitone. Even though we could record PB-elicited currents at pH 9.1, there is no certainty on whether the receptors were activated by charged or neutral forms of PB. We are unable to rule out a possibility that at pH 9.1, the receptors are activated by the neutral PB molecules while block is mediated by both charged and neutral species of PB.

A cluster of single channel activity at high concentrations of agonist is terminated when the receptor enters a long-lived desensitized state. For the $GABA_A$ receptor, in particular, shorter-lived desensitized states occur and may contribute to the intracluster closed duration distributions. It has been

shown for nicotinic ACh receptors that at high agonist concentrations (at high P_o) the cluster durations are reduced as a result of desensitization. It was proposed that the receptors enter the long-lived desensitized state only from the open state, thus the sum of all open interval durations per cluster is constant (Auerbach & Akk, 1998). In experiments with GABA + PB, we found that even though the P_o is greatly reduced when the PB concentration is over 500 μM, this is not accompanied by an increase in the cluster duration (Table 2). This observation indicates that blockade does not prevent desensitization. This suggests that open and blocked channels desensitize at a similar rate.

J.H. Steinbach is the Russell and Mary Shelden Professor of Anaesthesiology. We thank John Bracamontes and Jessie Zhang for advice and assistance with experiments. Supported by P01 GM 47969 to J.H. Steinbach.

References

AKAIKE, N., HATTORI, K., INOMATA, N. & OOMURA, Y. (1985). γ-Aminobutyric-acid- and pentobarbitone-gated chloride currents in internally perfused frog sensory neurones. *J. Physiol. (London),* **360,** 367 – 386.

AKAIKE, N., MARUYAMA, T. & TOKUTOMI, N. (1987). Kinetic properties of the pentobarbitone-gated chloride current in frog sensory neurones. *J. Physiol. (London),* **394,** 85 – 98.

AKAIKE, N., TOKUTOMI, N. & IKEMOTO, Y. (1990). Augmentation of GABA-induced current in frog sensory neurons by PB. *Am. J. Physiol.,* **258,** C452 – C460.

AMIN, J. (1999). A single hydrophobic residue confers barbiturate sensitivity to γ-aminobutyric acid type C receptor. *Mol. Pharm.,* **55,** 411 – 423.

AMIN, J. & WEISS, D.S. (1993). $GABA_A$ receptor needs two homologous domains of the β-subunit for activation by GABA but not by pentobarbitone. *Nature,* **366,** 565 – 569.

AUERBACH, A. & AKK, G. (1998). Desensitization of mouse nicotinic acetylcholine receptor channels. A two-gate mechanism. *J. Gen. Physiol.,* **112,** 181 – 197.

AUSUBEL, F.M., BRENT, R., KINGSTON, R.E., MOORE, D.D., SEIDMAN, J.G., SMITH, J.A. & STRUHL, K. (1992). *Short Protocols in Molecular Biology*. John Wiley & Sons, New York.

BELELLI, D., PISTIS, M., PETERS, J.A. & LAMBERT, J.J. (1999). General anaesthetic action at transmitter-gated inhibitory amino acid receptors. *Trends in Pharmacol. Sci.,* **20,** 496 – 502.

CESTARI, I.N., UCHIDA, I., LI, L., BURT, D. & YANG, J. (1996). The agonistic action of pentobarbitone on $GABA_A$ β-subunit homomeric receptors. *NeuroReport,* **7,** 943 – 947.

COLQUHOUN, D. & SIGWORTH, F.J. (1995). Fitting and statistical analysis of single-channel records. In *Single-channel recording*. ed. Sakmann, B. & Neher, E. pp. 483 – 587. Plenum Press, New York and London.

COTTRELL, G.A., LAMBERT, J.J. & PETERS, J.A. (1987). Modulation of GABAA receptor activity by alphaxalone. *Br. J. Pharmacol.,* **90,** 491 – 500.

DAVIES, P.A., KIRKNESS, E.F. & HALES, T.G. (1997). Modulation by general anaesthetics of rat $GABA_A$ receptors comprised of $\alpha1\beta3$ and $\beta3$ subunits expressed in human embryonic kidney 293 cells. *Br. J. Pharmacol.,* **120,** 899 – 909.

DILGER, J.P., BOGUSLAVSKY, R., BARANN, M., KATZ, T. & VIDAL, A.M. (1997). Mechanisms of barbiturate inhibition of acetylcholine receptor channels. *J. Gen. Physiol.,* **109,** 401 – 414.

FFRENCH-MULLEN, J.M., BARKER, J.L. & ROGAWSKI, M.A. (1993). Calcium current block by (−)-pentobarbitone, phenobarbital, and CHEB but not (+) pentobarbitone in acutely isolated hippocampal CA1 neurons: comparison with effects on GABA-activated Cl-current. *J. Neurosci.,* **13,** 3211 – 3221.

GAGE, P.W. & MCKINNON, D. (1985). Effects of pentobarbitone on acetylcholine-activated channels in mammalian muscle. *Br. J. Pharmacol.,* **85,** 229 – 235.

HAMILL, O.P., MARTY, A., NEHER, E., SAKMANN, B. & SIGWORTH, F.J. (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Arch.,* **391,** 85 – 100.

HORN, R. (1987). Statistical methods for model discrimination. Applications to gating kinetics and permeation of the acetylcholine receptor channel. *Biophys. J.,* **51,** 255 – 263.

JACKSON, M.B., LECAR, H., MATHERS, D.A. & BARKER, J.L. (1982). Single channel currents activated by γ-aminobutyric acid, muscimol and (−)-pentobarbitone in cultured mouse spinal neurons. *J. Neurosci.,* **2,** 889 – 894.

KRISHEK, B.J., AMATO, A., CONNOLLY, C.N., MOSS, S.J. & SMART, T.G. (1996). Proton sensitivity of the $GABA_A$ receptor is associated with the receptor subunit composition. *J. Physiol.,* **492,** 431 – 443.

LAMBERT, J.J., BELELLI, D., PISTIS, M., HILL-VENNING, C. & PETERS, J.A. (1997). The interaction of intravenous anesthetic agents with native and recombinant $GABA_A$ receptors. In *The GABA receptors*. ed. Enna, S.J. & Bowery, N.G. pp. 121 – 156. Humana Press, Totowa, New Jersey.

LEVITAN, E.S., BLAIR, L.A.C., DIONNE, V.E. & BARNARD, E.A. (1988). Biophysical and pharmacological properties of cloned $GABA_A$ receptor subunits expressed in Xenopus oocytes. *Neuron,* **1,** 773 – 781.

MACDONALD, R.L., ROGERS, C.J. & TWYMAN, R.E. (1989). Barbiturate regulation of kinetic properties of the $GABA_A$ receptor channel of mouse spinal neurones in culture. *J. Physiol. (London),* **417,** 483 – 500.

MACONOCHIE, D.J., ZEMPEL, J.M. & STEINBACH, J.H. (1994). How quickly can $GABA_A$ receptors open? *Neuron,* **12,** 61 – 71.

MATHERS, D.A. & BARKER, J.L. (1980). (−)Pentobarbitone opens ion channels of long duration in cultured mouse spinal neurons. *Science,* **209,** 507 – 509.

NEHER, E. & STEINBACH, J.H. (1978). Local anaesthetics transiently block currents through single acetylcholine receptor channels. *J. Physiol. (London),* **277,** 153 – 176.

NICOLL, R.A. (1975). Pentobarbitone: action on frog motoneurons. *Brain Res.,* **96,** 119 – 123.

OLSEN, R.W. & TOBIN, A.J. (1990). Molecular biology of $GABA_A$ receptors. *FASEB J.,* **4,** 1469 – 1480.

PISTIS, M., BELELLI, D., PETERS, J.A. & LAMBERT, J.J. (1997). The interaction of general anaesthetics with recombinant $GABA_A$ and glycine receptors expressed in *Xenopus laevis* oocytes: a comparative study. *Br. J. Pharmacol.,* **122,** 1707 – 1719.

QIN, F., AUERBACH, A. & SACHS, F. (1996). Estimating single-channel kinetic parameters from idealized patch clamp data containing missed events. *Biophys. J.,* **70,** 264 – 280.

QIN, F., AUERBACH, A. & SACHS, F. (1997). Maximum likelihood estimation of aggregated Markov processes. *Proc. R. Soc. B,* **264,** 375 – 383.

RHO, J.M., DONEVAN, S.D. & ROGAWSKI, M.A. (1996). Direct activation of $GABA_A$ receptors by barbiturates in cultured rat hippocampal neurons. *J. Physiol. (London),* **497,** 509 – 522.

ROBERTSON, B. (1989). Actions of anaesthetics and avermectin on $GABA_A$ chloride channels in mammalian dorsal root ganglion neurones. *Br. J. Pharmacol.,* **98,** 167 – 176.

SAKMANN, B., PATLAK, J. & NEHER, E. (1980). Single acetylcholine-activated channels show burst-kinetics in presence of desensitizing concentrations of agonist. *Nature,* **286,** 71 – 73.

TAVERNA, F.A., CAMERON, B.R., HAMPSON, D.L., WANG, L.Y. & MACDONALD, J.F. (1994). Sensitivity of AMPA receptors to PB. *Eur. J. Pharmacol.,* **267,** R3 – R5.

THOMPSON, S.A., WHITING, P.J. & WAFFORD, K.A. (1996). Barbiturate interactions at the human $GABA_A$ receptor: dependence on receptor subunit combination. *Br. J. Pharmacol.,* **117,** 521 – 527.

UPTON, N. & BLACKBURN, T. (1997). Pharmacology of mammalian $GABA_A$ receptors. In *The GABA receptors* (eds) Enna SJ and Bowery NG. pp. 83 – 120, Humana Press, Totowa, New Jersey.

WOOLTORTON, J.R., MOSS, S.J. & SMART, T.G. (1997). Pharmacological and physiological characterization of murine homomeric beta3 GABA(A) receptors. *Eur. J. Neurosci.,* **9,** 2225–2235.

YAMAKURA, T., SAKIMURA, K., MISHINA, M. & SHIMOJI, K. (1995). The sensitivity of AMPA-selective glutamate receptor channels to pentobarbitone is determined by a single amino acid residue of the alpha 2 subunit. *FEBS Lett.,* **374,** 412–414.

(Received November 16, 1999
Revised January 25, 2000
Accepted February 29, 2000)