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Kinetic analysis of the agonistic and blocking properties of pentobarbital on recombinant rat $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$ receptor channels

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Abstract

Barbiturates have three different effects on the $GABA_A$ receptor channels: coactivation, direct activation, and blockage. We investigated the activation and blockage of the $GABA_A$ receptor channels by pentobarbital using the $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$ receptor channels transiently expressed in HEK293 cells in combination with the ultrafast application of agonists. The peak current amplitude of the pentobarbital activated ionic current proportionally increased to the first power of the pentobarbital concentration (Hill coefficient ~ 0.7), indicating that one binding step of pentobarbital at $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$ receptor channels can describe the experimental dose–response relation. The maximum peak current amplitude occurred at 1 mM pentobarbital and decreased at higher concentrations due to an open channel block. After the end of the pentobarbital pulses, rebound currents due to transition from the open-blocked to the open state of the receptor were observed. A kinetic scheme was constructed allowing the quantitative analysis of the pentobarbital activated ionic currents through $GABA_A$ receptor channels.

Keywords: $GABA_A$ receptor; Pentobarbital; Fast application; Computer simulation

1. Introduction

$GABA_A$ receptor channels are pentamers made up of combinations of different subunits, forming a central pore selectively permeable to Cl^- ions. The $\alpha_1\beta_2\gamma_2$ receptor subtype is found in most brain areas (Mohler et al., 1996). Barbiturates are widely used as anticonvulsive, hypnotic, and anesthetic drugs. It is generally accepted that they have three different mechanisms of action at $GABA_A$ receptor channels: (1) potentiation of the current response elicited by GABA (Evans, 1979); (2) agonistic effects at $GABA_A$ receptor channels (Franks and Lieb, 1994; Robertson, 1989); and (3) blockage of the $GABA_A$ receptor channel currents (Peters et al., 1988; Robertson, 1989). Additionally, interactions of barbiturates with voltage-activated Ca^{2+} currents (Ffrench-Mullen et al., 1993), voltage-dependent potassium channels (Friedrich and Urban, 1999), AMPA and kainate type glutamate receptors (Taverna et al., 1994; Dildy-Mayfield et al., 1996), and nicotinic receptor channels (Krampfl et al., 2000b) were reported.

Previously, it was shown that pentobarbital-induced single-channel openings have the same slope conductance as the GABA-activated single-channel currents (Mathers and Barker, 1980; Rho et al., 1996). Pentobarbital-induced single-channel openings can be blocked by antagonists of $GABA_A$ receptor channels (Rho et al., 1996). Mutagenesis experiments revealed that the mutant $GABA_A$ receptor channels with up to 900-fold lower affinity for GABA have an unchanged affinity for pentobarbital (Amin and Weiss, 1993). This indicates that the pentobarbital binding site is different from the GABA binding site at the $GABA_A$ receptor channel protein.

Concentration clamp experiments using tools for ultrafast solution exchange in combination with the patch clamp technique allow direct measurement of molecular reactions between ligands and receptors. The kinetics of ensemble currents after the application of pentobarbital to recombinant rat $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$ receptor channels were investigated to construct a molecular scheme for the activation and blockage of the $GABA_A$ receptor channels by pentobarbital. Using such molecular schemes, the drug–receptor interactions can be predicted by means of computer simulation (Bufler et al., 1996a,b). This approach was used in the present study for the quantitative analysis of the direct

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effects of pentobarbital on recombinant $GABA_A$ receptor channels.

2. Materials and methods

2.1. Expression of recombinant $GABA_A$ receptors

Transformed human embryonic kidney (HEK) 293 cells were cultured in DMEM, supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in a 5% CO_2/95% air incubator. Cells were suspended in a buffer used for transfection (in mM: 50 $K_2HPO_4 \cdot 3H_2O$, 20 K−acetate, pH 7.35). For transient co-transfection of rat α_1, β_2, and γ_{2S} $GABA_A$-receptor subunits, the corresponding cDNAs, subcloned each in pcDM8 expression vector (Invitrogen), were added to the suspension. Plasmid DNAs for the three $GABA_A$-receptor subunits were used in a ratio of 1:1:2 for $\alpha_1/\beta_2/\gamma_{2S}$. For transfection, we used an electroporation device by EquiBio (UK). Transfected cells were replated on glass coverslips in DMEM containing 10% FCS and incubated for 15−18 h.

2.2. Electrophysiology

Patch-clamp measurements were performed in the cell attached mode for single-channel recordings and with the outside-out patches for fast application experiments using standard methods (Hamill et al., 1981). Patch pipettes were pulled from borosilicate glass tubes with a DMZ-Universal Puller (Zeitz Instruments, Augsburg, Germany). They had a series resistance between 5 and 10 MΩ when filled with intracellular solution containing (in mM) 140 KCl, 11 EGTA, 10 Hepes, 10 glucose, 2 $MgCl_2$. The osmolarity was adjusted to 340 mosm l^{-1} with mannitol. HEK293 cells were superfused with an extracellular solution containing (in mM) 162 NaCl, 5.3 KCl, 0.67 $NaHPO_4$, 0.22 KH_2PO_4, 15 Hepes, 5.6 glucose. The pH of both solutions was adjusted to 7.3. Data were recorded with an Axopatch200B patch-clamp amplifier (Axon Instruments, Foster City, CA, USA). The holding potential was at −60 mV if not otherwise stated.

2.3. Ultrafast solution exchange

Ultrafast application of agonists to outside-out patches was performed using a piezo-driven device (Franke et al., 1987), allowing the application and de-application times of well defined agonist concentrations of < 100 μs and 0.5 ms, respectively (Bufler et al., 1996a; Heckmann et al., 1996). The core of the application device is a single outlet glass tubing connected to a reservoir for test solutions which are driven through the outlet by air pressure and form a laminar flowing liquid filament. The glass tubing is mounted on a piezo translator allowing for the ultrafast switching between two positions by a 20-μm extension elicited by the application of a current pulse. The tip of the glass electrode with the adhering membrane patch is placed next to the laminar liquid filament formed by the test solution. For the application of the test solution, the liquid filament is placed to superfuse the tip of the electrode with the membrane patch. The performance of the system was tested by switching the ionic concentration (1:10 with distilled water diluted extracellular solution) at the tip of the open patch pipettes and measuring the change in the liquid junction current. The time course of the solution exchange was fitted with a single exponential (time constant 53 μs) in the experiment shown in Fig. 1.

2.4. Kinetic analysis

Ensemble currents were sampled with 10 kHz to the hard disk of a PC using a Digidata 1200 Interface and the pCLAMP6 software suit (Axon Instruments). For further analysis, data were filtered at 2 kHz. Multiple (10−20) current traces were recorded for each pentobarbital concentration at 30-s intervals and averaged for analysis. Intervals of 30 s were necessary to minimize the rundown or accumulation of $GABA_A$ receptors in desensitized or blocked states. Experimental protocols found to be most effective were alternating applications of 1 mM GABA (five single-current traces) and the pentobarbital concentrations tested. Since the application protocols with GABA-pulses at the length of seconds lead to the accumulation of channels in desensitized states and rundown effects lead to a further loss of excitable channels in the course of a experiment, we increased the intervals between single applications in this study beginning with a value of 10 s. While intervals between pulses as long as 30 s were most sufficient to avoid rundown after the solution exchange (between GABA and pentobarbital), the interval was extended to 2 min to rule out GABA and pentobarbital mutually influencing the current kinetics by allosteric modulation. All experimental data are given as mean ± SD.

GABA was obtained from Sigma (St. Louis, MO, USA). Pentobarbital was obtained from Synopharm (Barsbüttel,

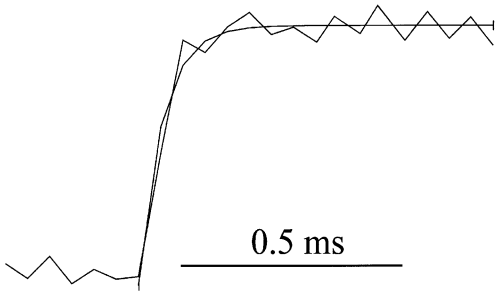
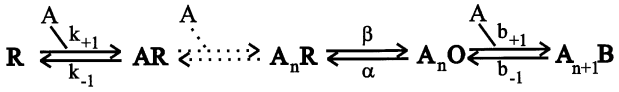


Fig. 1. Time course of solution exchange by the piezo-driven liquid filament switch. The bathing solution passing an open pipette tip was changed from the extracellular solution to a solution with 90% reduced Na^+. The smooth curve shows the time course of solution exchange fitted monoexponentially.



Scheme 1.

Germany). Solutions were freshly prepared before each experiment.

2.5. Modeling

The calculated responses of various reaction models were obtained by a further developed version of a program (Parnas et al., 1989) which solves sets of differential equations valid for special kinetic schemes iteratively for short intervals of time. The fourth-order of the Runge–Kutta method of integration was used. The solution of the differential equations defines the time course of the different states of the molecular reaction scheme (Scheme 1) before, during, and after the application of a pulse of pentobarbital. The calculations were performed on personal computers.

3. Results

To analyze single-channel properties, measurements were performed in the cell-attached mode of the patch-clamp technique with 0.07 mM pentobarbital added to the extracellular solution of the patch electrode. Under these conditions, single-channel openings, partly disrupted by short closings, were observed (Fig. 2A). At a holding potential of +100 mV, the single-channel amplitude revealed a main conductance state of around 3 pA (Fig. 1B; mean 2.8 ± 0.08 pA, 1597 events in four different patches). The single-channel slope conductance was 28 pS, very close to that of the GABA-activated single-channel currents measured from the same $GABA_A$ receptor subtype (Jahn et al., 1997). The mean open time and the mean closed time within bursts of single-channel openings were 5.3 ± 2.2 and 0.35 ± 0.12 ms, respectively ($n = 4$, analysis of 1597 events in four different patches; see the representative experiment in Fig. 1C).

Fast application of GABA to outside-out patches of HEK293 cells containing $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$ receptor channels resulted in a Cl^--current reaching the maximum amplitude at 1 mM GABA (Jahn et al., 1997; Krampfl et al., 2000a). Fig. 3 shows a current following a 200-ms pulse of 1 mM GABA to an outside-out patch. The current rose to the peak amplitude of −88.6 pA within 0.4 ms and desensitized incompletely, best fitted with three time constants to a steady state current of 13% in this experiment. In contrast, currents of $\alpha_1\beta_2$ receptor channels desensitized with two time constants to lower steady state values (Haas and Macdonald, 1999; Krampfl et al., 2000a). It was therefore concluded that $GABA_A$ receptor channels containing the γ_{2S}-subunit were expressed (Krampfl et al., 2000a).

Upon the application of pentobarbital to the outside-out patches from HEK293 cells expressing the $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$ receptor channels, ensemble currents with kinetics different from that of the GABA-activated currents were observed. Experiments of Figs. 3 and 4A were performed at the same patch. Pentobarbital was applied with pulses of 200-ms duration. Application of 0.1 mM pentobarbital resulted in a small nondesensitizing current (−3.7 pA, upper trace,

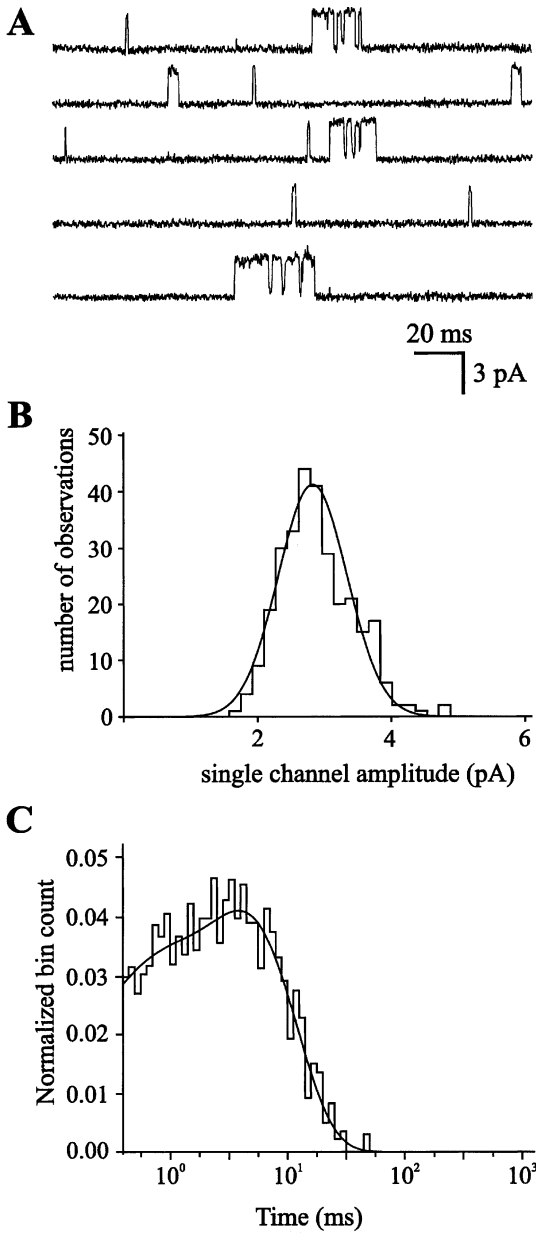


Fig. 2. (A) Single channel measurements in the cell-attached mode; 0.07 mM pentobarbital was added to the pipette solution. Holding potential was +100 mV. (B) Amplitude histogram of pentobarbital-activated single-channel currents of the patch shown in (A). (C) Logarithmically binned open time frequency distribution for unitary currents evoked by 0.07 mM pentobarbital. The histogram is derived from data from a single cell attached patch. The histogram was fitted to a second-order exponential function with the time constants $\tau_1 = 0.41$ ms and $\tau_2 = 4.67$ ms.

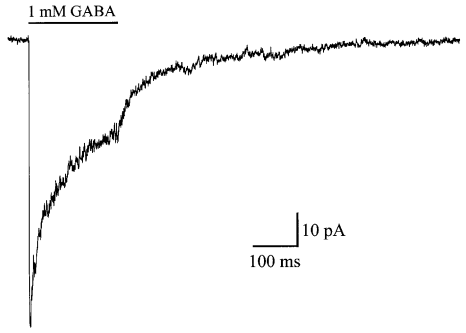


Fig. 3. Macroscopic current activated by 1 mM GABA applied for 200 ms to an outside-out patch containing $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$ receptor channels. The current trace is the average of three single-current traces. Holding potential was -60 mV.

Fig. 4A). The current reached a maximum amplitude at 1 mM pentobarbital (-21.2 pA, middle trace, Fig. 4A) added to the extracellular solution. At higher pentobarbital concentrations, the peak current amplitude decreased and reached -5.0 pA when 10 mM pentobarbital was applied (Fig. 4A, lower trace). In contrast to $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$ receptor channel currents activated by GABA, no desensitization of the pentobarbital activated current was observed (Fig. 4). The 10–90% rise time of the current decreased with increasing pentobarbital concentrations and reached 3.6 ms (mean 5.4 ± 1.8 ms ($n=5$)) when 10 mM pentobarbital was added to the test solution (Fig. 4A). At concentrations ≤ 0.1 mM pentobarbital, currents decayed after cessation of the pentobarbital application due to dissociation of pentobarbital from the receptor (Fig. 4, upper trace). However, pentobarbital concentrations ≥ 0.3 mM resulted in a rebound current after the end of pentobarbital pulses (Fig. 4A, middle and lower trace). The peak current amplitudes after the end of pentobarbital pulses were considerably higher than after the application of pentobarbital (-52.5 pA at 1 mM pentobarbital and -77.5 pA at 10 mM pentobarbital in the experiment of Fig. 4A).

In Fig. 4B, an experiment with 2-s pulses of 10 mM pentobarbital was shown to demonstrate that the pentobarbital activated current has no desensitizing component. The rise time of the rebound current could be fitted with a single exponential (see onset of the rebound current on an expanded time scale, inset of Fig. 4B), was concentration independent and had a value of 4.7 ms (see inset of Fig. 4B; mean 4.7 ± 0.5 ms ($n=5$)).

The diagrams in Fig. 5 summarize the dose–response curves of five independent experiments. The peak current amplitude activated by pentobarbital and the amplitude of the rebound current were normalized to the peak current amplitude measured with 1 mM GABA (as described by Thompson et al., 1996) as $p_{o,\ peak}$ and $p_{o,\ rebound}$, respectively. The $p_{o,\ peak}$ was 0.03 ± 0.02 ($n=5$) at 0.03 mM pentobarbital and increased to 0.39 ± 0.15 ($n=5$) at 1 mM pentobarbital. At higher concentrations, $p_{o,\ peak}$ decreased and had a value of 0.03 ± 0.02 ($n=4$) at 10 mM pentobarbital due to the blockage of $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$ receptor channels by pentobarbital (Fig. 5A). The slope of the dose–response curve was ~ 0.7 (Hill coefficient) between 0.03 and 0.3 mM pentobarbital in a double logarithmic plot (see inset of Fig. 5A).

In Fig. 5B, evaluation of rebound currents was performed. When 0.3 mM pentobarbital was added to the test solution, $p_{o,\ rec}$ was 0.38 ± 0.13 ($n=4$) and reached a maximum value of 0.86 ± 0.18 ($n=4$) with 3 mM pentobarbital added to the test solution.

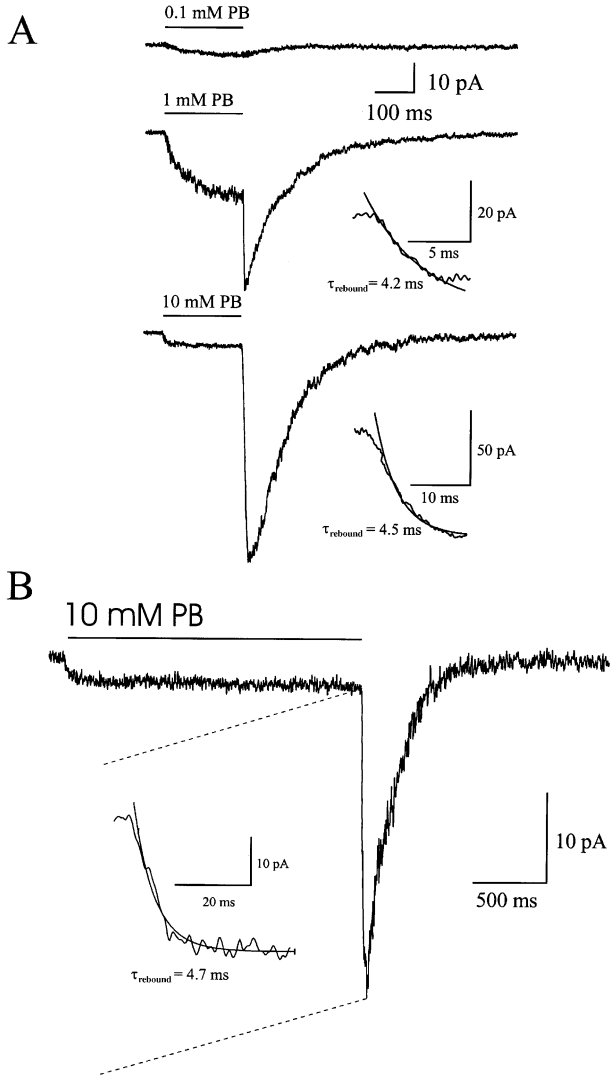


Fig. 4. (A) Dose–response experiments for currents activated by different concentrations of pentobarbital, as indicated. Pentobarbital was applied for 200 ms. The same outside-out patch as in Fig. 3 was used for the experiment. The insets next to the middle and bottom trace show the rising phase of the rebound current with an expanded time scale (as indicated by dotted lines in (B)). The smooth curves show the overlay of the monoexponential fit of the current rise after the offset of pentobarbital. (B) 10 mM pentobarbital was applied for 2 s. The inset shows the onset of the rebound current with an expanded time scale. The smooth curve shows the monoexponential fit with a time constant ($\tau_{rebound}$) of 4.7 ms. Each trace is the average of at least three single-current traces. Holding potential was -60 mV.

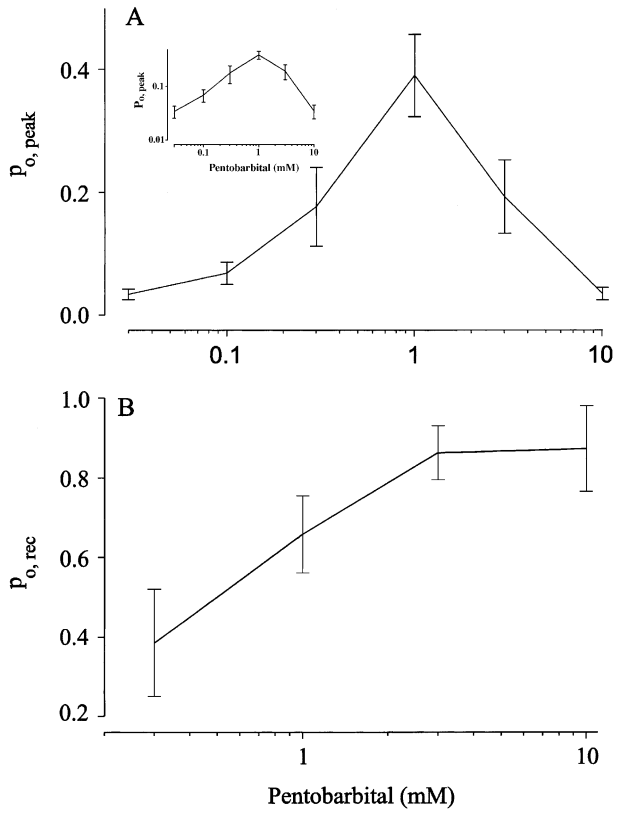
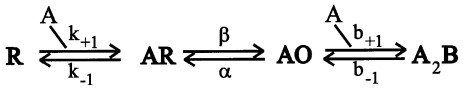


Fig. 5. Dose–response diagrams for the currents activated by application of pentobarbital in different concentrations. (A) Dose–response curve of the relative peak current amplitude ($p_{o,\ peak}$) after application of different pentobarbital concentrations. The inset shows the dose–response curve in a double logarithmic diagram. (B) Relative peak current amplitude of the rebound current ($p_{o,\ rec}$). Each point summarizes at least four independent experiments.

Computerized calculations allow kinetic analysis of receptor channels with quantitative determination of the rate constants in specific kinetic schemes and predictions about the behavior of the channels under various experimental conditions. Scheme 1 was adopted from Akaike et al. (1987). In this scheme, R is the unliganded closed receptor, A is the agonist, O is the open state, and B is the blocked state of the receptor. The Hill coefficient gives the minimum number of agonist molecules binding to the receptor. The experimental dose–response curves in our experiments could not be fitted with the Hill equation because it was obscured by the open channel block at higher pentobarbital concentrations similar to the data shown by Akaike et al. (1987). However, the slope of the dose–response curve at low concentrations also determines the lower limit of the number of binding agonist molecules necessary for the channel opening (Katz and Thesleff, 1957; Dreyer et al., 1978; Colquhoun and Ogden, 1988). At pentobarbital concentrations between 0.03 and 0.1 mM, rebound currents reflecting the transition from the blocked state to the open state of the channels could not be observed (Fig. 4A). The slope of the dose–response curve in the double logarithmic Hill plot at such low concentrations was 0.7 (inset of Fig. 5A), predicting that the binding of one pentobarbital molecule at $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$ receptor channels can generate the observed dose–response relation. Thus, the dose–response curve can be modeled assuming a single activating binding site (Scheme 2). In this scheme, the unliganded closed R state of the receptor is connected with the single liganded AR state via the binding rate constant k_{+1} and the unbinding rate k_{-1}. From the AR state, the receptor changes the conformation to the open state of the receptor (AO) via the concentration independent isomerization rates β and α. The AO state is linked with the A_2B state after the binding of a second agonist molecule via b_{+1} and b_{-1}.

The affinity of the agonist to the activating site of the receptor channels is determined by the binding rate constant k_{+1} and the unbinding rate constant k_{-1} (Scheme 2). The mean open time of the channels depends on α. From the mean open time within bursts of 5.3 ms (Fig. 2), a value of $\alpha \approx 200\ s^{-1}$ was estimated. The onset of the rebound current could be fitted monoexponentially (see inset of Fig. 4B) and is described by the transition from the blocked (A_2B) to the open state (AO) of the receptor in Scheme 2. Under these conditions, b_{-1} is the reciprocal of the time constant of the rebound current ($\sim$5 ms), resulting in a concentration-independent unblocking rate $b_{-1} = 200\ s^{-1}$. Experimentally, $p_{o,\ rec}$ at 1 mM pentobarbital was around twice that of $p_{o,\ peak}$ (Fig. 4A, middle trace; Fig. 5), i.e., the probability of the A_2B state being occupied is equal to that of the AO state. Therefore, at 1 mM pentobarbital, b_{+1}, and b_{-1} have about the same values. The unblocking rate b_{-1} was $200\ s^{-1}$; the value of b_1 was therefore estimated as $2 \times 10^5\ M^{-1}s^{-1}$ (resulting in a blocking rate $b_{+1} = 200\ s^{-1}$ at 1 mM pentobarbital). The rate constants for k_{+1}, k_{-1}, and β were determined by trial and error.

In Fig. 6, the time course of the open state of $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$ receptor channels was calculated with the following rate constants according to Scheme 2: $k_{+1} = 8000\ M^{-1}\ s^{-1}$, $k_{-1} = 800\ s^{-1}$, $b_{+1} = 200\,000\ M^{-1}\ s^{-1}$, $b_{-1} = 200\ s^{-1}$, $\beta = 20\,000\ s^{-1}$, $\alpha = 200\ s^{-1}$. The calculated time course of the open state of the receptor ($p_{o,\ sim}$) fits the experimental data quite well. The probability of the open state being occupied ($p_{o,\ sim}$) reached 0.09 after application of 0.1 mM pentobarbital, 0.33 at 1 mM pentobarbital, and 0.09 at 10 mM pentobarbital. The bell-shaped (Akaike et al., 1987) experimental dose–response curve could be predicted by the calculated curves. Kinetic schemes with more than one binding site (Scheme 1; Akaike et al., 1987; Wooltorton et al., 1997) resulted in a considerably steeper dose–response curves at low agonist concentrations (not shown). After the end of pentobarbital pulses, rebound currents were



Scheme 2.

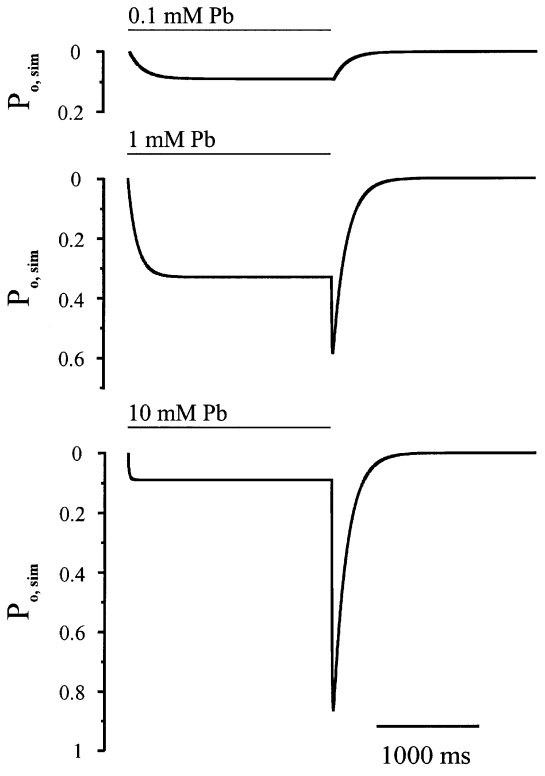


Fig. 6. Computer-generated time courses of the probabilities (ordinates) of the AO-state after application of 0.1, 1, and 10 mM pentobarbital for 2 s; solutions based on Scheme 2. For the calculations, the following values were used (see Results and Discussion): $k_{+1}=8000\ M^{-1}\ s^{-1}$, $k_{-1}=800\ s^{-1}$, $b_{+1}=200\,000\ M^{-1}\ s^{-1}$, $b_{-1}=200\ s^{-1}$, $\beta=20\,000\ s^{-1}$, $\alpha=200\ s^{-1}$.

obtained with kinetics similar to that observed experimentally. At 10 mM pentobarbital, most of the receptors were shifted to the A_2B state of Scheme 2. Recovery from the blocked A_2B state of the receptor proceeded exclusively via the AO state and not, as shown for the open channel blockage of nicotinic receptors (Bufler et al., 1996a,b; Krampfl et al., 2000b), via desensitized blocked states. After the end of pentobarbital pulses ≥ 0.3 mM, the AO state of the receptor is filled up with the time course given by the unbinding rate b_{-1}, resulting in rebound currents. The relative amplitudes of $p_{o,\,sim}$ corresponded quite well to that of the experimental dose–response curves of the rebound currents (Figs. 5B and 6). A systematic deviation of the calculated curves from the experimental dose–response curve was that the relative experimental current amplitude at 10 mM pentobarbital ($p_{o,\,peak}$) was lower than the calculated $p_{o,\,sim}$. This mismatch could not be resolved by the variation of the rate constants in Scheme 2. Higher values of b_{+1} or lower values of b_{-1} resulted in too low values of $p_{o,\,sim}$ at 1 mM pentobarbital.

4. Discussion

The single-channel slope conductance of pentobarbital activated single-channels in the present study was very near to that of the main conductance state of GABA activated single-channel currents at the same recombinant $GABA_A$ receptor channels (Jahn et al., 1997). Single-channel analysis at cultured rat pyramidal hippocampal neurons showed that the current activated by pentobarbital was carried by Cl^- and could be antagonized by picrotoxin. The mean open time and the closed time within bursts of single-channel currents in the present study were in the same range as that of the native $GABA_A$ receptors (Rho et al., 1996).

Rebound currents after the end of pulses of pentobarbital application were found in most studies investigating the direct effects of pentobarbital on $GABA_A$ receptor channels (Akaike et al., 1987; Dalziel et al., 1999; Rho et al., 1996; Thompson et al., 1996; Wooltorton et al., 1997). However, there are profound differences relative to the current decay of $GABA_A$ receptor channels in the presence of pentobarbital. Desensitization of recombinant $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$ receptor channels after the fast application of 1 mM GABA was best fitted with three time constants (Fig. 3; Haas and Macdonald, 1999; Krampfl et al., 2000a). In contrast, $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$ receptor channels responded with non-desensitizing currents upon the application of pentobarbital to the outside-out patches (Fig. 4B). Dalziel et al. (1999) and Rho et al. (1996) only observed slight desensitizing currents upon the application of pentobarbital to whole cells containing recombinant $\alpha_1\beta_1$ $GABA_A$ receptor channels or native $GABA_A$ receptor channels from hippocampal neurons, respectively, whereas Akaike et al. (1987) showed distinct desensitization especially at high pentobarbital concentrations with intracellular measurements on native $GABA_A$ receptor channels of frog sensory neurons. This study may serve as a representative case for the whole cell current under natural conditions. Similarly, Wooltorton et al. (1997) described desensitization at high pentobarbital concentrations with whole cell registrations on recombinant homomeric β_3 $GABA_A$ receptor channels. The reason for that difference may arise from different receptors investigated and/or different measurement techniques. In our study, which was focused on the properties of one specific $GABA_A$ receptor subtype, we used the outside-out patches in combination with a piezo-driven application system, which allows a good control of the solution exchange (Fig. 1) and observed no current decay upon the application of pentobarbital in the concentration range tested. When pentobarbital was applied to whole cells in our experiments, slight desensitization of currents similar to the data of Akaike et al. (1987) or Dalziel et al. (1999) was observed (not shown). Therefore, slight desensitization of the $GABA_A$ receptor channels upon the application of pentobarbital might be due to the application techniques of pentobarbital to whole cells, whereas the massive desensitization in the papers of Rho et al. (1996) and Wooltorton et al. (1997) might reflect different properties of the respective receptor channels investigated.

When the dose–response curve was normalized to the maximum current (Thompson et al., 1996) after the appli-

cation of 1 mM GABA, $p_{o,\,peak}$ was 0.4 after the application of 1 mM pentobarbital (Fig. 5A). Thompson et al. (1996) found a higher relative maximum current response after the bath application of pentobarbital using the same recombinant $GABA_A$ receptor channels expressed in oocytes. One reason for that difference may be the underestimation of the amplitude of the GABA-activated currents because of slow agonist perfusion techniques to whole oocytes used in that study (desensitization of the fast desensitizing component of $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$ receptor channels was between 10 and 20 ms, carrying at least 10% of the whole current amplitude; Krampfl et al., 2000a; see also Haas and Macdonald, 1999).

The dose–response relation of pentobarbital activated currents was bell-shaped (Fig. 4A) with decreasing relative current amplitudes at concentrations >1 mM pentobarbital. The relative current amplitude at 10 mM pentobarbital was as small as that after the application of 0.1 mM pentobarbital (Fig. 5A). This may be due to an additional blocking site of pentobarbital at $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$ receptor channels, resulting in an open channel block. Further evidence for a mechanism of the open channel blockage came from the rebound currents developing after the end of pentobarbital pulses >0.3 mM (Fig. 4B), similar to the ''rebound'' observed in previous studies (Akaike et al., 1987; Dalziel et al., 1999; Rho et al., 1996; Thompson et al., 1996; Wooltorton et al., 1997). At the end of pentobarbital pulses, the rise time of the rebound current could be properly measured. It was independent of the pentobarbital concentration and could be fitted monoexponentially with a time constant of $\sim$5 ms (see inset of Fig. 4B).

Pentobarbital-activated currents in our study were calculated using Scheme 2 with a single activating binding site for pentobarbital at the receptor. The main argument for the single binding site was the experimentally found slope of the dose–response curve at low pentobarbital-concentrations (Fig. 5A). As shown in Fig. 6, the bell-shaped dose–response curves could be quite well reproduced in the calculated traces of $p_{o,\,sim}$. The shape of the dose–response curve points to a higher affinity of pentobarbital to the activating site of the receptor. In Scheme 2, a second binding site of pentobarbital is at the open state of the channel. According to Scheme 2, since the pentobarbital binding at the closed state of the receptor is considerably slower than binding to the open state of the channel ($k_{+1}=8000\ M^{-1}\ s^{-1}$, $b_{+1}=2\times10^5\ M^{-1}\ s^{-1}$), the current amplitude decreased at high pentobarbital concentrations (bell-shaped dose–response curve; Akaike et al., 1987) and showed no fast current decay after binding of the agonist, as observed at the open channel block of the nicotinic receptor channels (Bufler et al., 1996a,b). The dose-dependency of the amplitude of the currents after the pentobarbital binding and of the rebound currents could be predicted very precisely by Scheme 2. Calculations were also performed with two binding sites of pentobarbital at the receptor (Scheme 1). Two binding sites would result in a slope of the dose–response curve >1 and a fast current decay in the presence of 10 mM pentobarbital due to a faster current rise time. Wooltorton et al. (1997) proposed a kinetic scheme with two binding sites of pentobarbital for channel activation and a third binding site for block. The kinetic scheme proposed in that paper was necessary to predict current decay in the presence of the agonist and rebound currents. The amplitude of the rebound current was dependent on the pulse duration in that paper. Therefore, a transition from the blocked to a blocked desensitized state had to be introduced. Because pentobarbital-activated currents of recombinant $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$ receptor channels showed no desensitization in the presence of pentobarbital until a concentration of 10 mM pentobarbital and the amplitude of the rebound current was not dependent on pulse duration (Fig. 4), we were able to predict the experimental results of our study with Scheme 2. However, the existence of further blocked states could not be ruled out.

During the clinical intravenous administration of pentobarbital, serum concentrations up to 200 μM pentobarbital were measured (Wermeling et al., 1987). Fifty percent of pentobarbital is protein-bound (Urban et al., 1995). Therefore, free drug concentrations can be estimated to 100 μM under therapeutic conditions. At these concentrations, $GABA_A$ receptors were significantly activated by pentobarbital, resulting in nondesensitizing ionic currents (Fig. 4). Therefore, the direct activation of Cl^- current responses probably contributes to the central nervous system depressant effects of pentobarbital as already suggested by Rho et al. (1996), whereas the open channel blockage of $GABA_A$ receptor channels at high pentobarbital concentrations in the millimolar (mM) range is clinically negligible.

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