Different Effects of Pentobarbital on Two γ-Aminobutyrate Receptors from Rat Brain: Channel Opening, Desensitization, and an Additional Conformational Change†

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ABSTRACT: The effect of pentobarbital on the responses of the γ-aminobutyric acid (GABA) receptor from rat brain was studied in quantitative measurements of GABA-mediated chloride-exchange rates (reflecting channel-opening equilibrium) and receptor desensitization rates by using $^{36}Cl^-$ tracer ion with native membrane vesicles. Pentobarbital effected the two phases of $^{36}Cl^-$ influx in different ways, supporting previous evidence that these are mediated by two different receptors [Cash, D. J., & Subbarao, K. (1987) *Biochemistry 26*, 7556; Cash, D. J., & Subbarao, K. (1987) *Biochemistry 26*, 7562]. Both the chloride-exchange rate and the desensitization rate of the faster desensitizing receptor were increased by pentobarbital at concentrations above 20 μM by an allosteric effect shifting the response curve to lower GABA concentrations. A similar enhancement of the responses of the slower desensitizing receptor occurred up to 200 μM pentobarbital. Two pentobarbital effector sites were involved in the allosteric mechanism. Above 500 μM pentobarbital, both the initial chloride-exchange rate and the desensitization rate of the slower desensitizing receptor were decreased. This inhibition, which was immediate, occurred with saturating as well as low GABA concentrations and therefore was not attributed to decreased GABA binding but to inhibitory sites for pentobarbital, different from the allosteric activating sites and the GABA binding sites. The chloride ion exchange activity was seen to recover with time, at concentrations above 1000 μM pentobarbital, in a process with a very steep dependence on pentobarbital concentration. This reactivation was attributed to the conversion of an initial form of the receptor to a final form that was less inhibited by pentobarbital. The similarity of the effects of pentobarbital on the chloride ion exchange with its effects on electrophysiological measurements supports the fact that these different techniques study the same phenomena. Comparisons of the effects of pentobarbital on desensitization and on high-affinity ligand binding measurements suggest that increased GABA binding at equilibrium reflects an increased conversion to the desensitized state.

A major effect of the widely used drug pentobarbital is its enhancement of GABA-ergic neurotransmission, which increases the inhibitory signals to the neurons. The response of the γ-aminobutyric acid (GABA) receptor, which mediates transmembrane chloride diffusion, is enhanced (Olsen, 1982; Johnston et al., 1984; Enna & Gallagher, 1983). Pentobarbital can be used as an anesthetic, sedative, or hypnotic drug and is a widely studied example of the barbiturate class. The GABA receptor complex, including the chloride channel, is also the site of action of the widely used benzodiazepine class of drugs. It is the major inhibitory neurotransmitter receptor in the mammalian brain.

The effect of pentobarbital on the response of the GABA receptor has been investigated by electrophysiological techniques. The effect of pentobarbital on the binding of GABA to the receptor has been investigated in equilibrium binding measurements. We are reporting a study of the effect of pentobarbital on the functional responses of the GABA receptor by measuring the open channel, as evidenced by the chloride ion exchange rate, using a membrane vesicle preparation prepared freshly from brain. The study is based on recent observations of GABA-mediated transmembrane chloride transfer (Subbarao & Cash, 1985; Allan et al., 1985; Harris & Allan, 1985) with membrane preparations. Pentobarbital- (Schwartz et al., 1984, 1985) and muscimol- (Schwartz et al., 1986a,b) dependent chloride translocation in a membrane preparation was also attributed to the GABA receptor. The study is based additionally on techniques for rapid measurements of ion flux, thereby resolving the rapid and often simultaneous processes of transmembrane ion exchange and receptor desensitization (Cash et al., 1985; Hess et al., 1983; Aoshima et al., 1981; Cash & Hess, 1980).

GABA receptor mediated chloride ion exchange in times of up to a few seconds was found to proceed in two phases limited by two phases of desensitization (Subbarao & Cash, 1985; Allan et al., 1985; Cash & Subbarao, 1987b). After removal of the faster desensitizing chloride-exchange activity by preincubation of the membrane with GABA, the remaining, slower desensitizing activity (second phase) could be studied alone and was shown to be described by processes of chloride exchange and desensitization that were kinetically first order (Cash & Subbarao, 1987a). The two phases were attributed to two receptors, one faster and one slower to desensitize, both on the same membrane (Cash & Subbarao, 1987c). By use of rapid mixing, quench-flow measurements, the processes of channel opening and desensitization could be resolved for these two similar receptors (Cash & Subbarao, 1987d).

In preliminary reports (Cash & Subbarao, 1987e, 1988) we reported that pentobarbital had different effects on chloride exchange mediated by the two receptors. This drug accelerated

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both desensitization and chloride exchange of both the receptors. However, the effect of higher concentrations of pentobarbital on the two phases of response was different. In times up to 1 s the slower phase of $^{36}Cl^-$ influx was completely abolished by 1000 μM pentobarbital while the amplitude of $^{36}Cl^-$ influx in the faster phase was practically unaffected. We now report the effect of pentobarbital on the channel opening of the two receptors, measured quantitatively by these techniques, over a wide (>100-fold) range of pentobarbital concentrations. The enhancement of channel opening with lower concentrations and its inhibition at higher concentrations of pentobarbital (for the slower desensitizing receptor) are similar to the effects of pentobarbital observed in electrophysiological experiments. In addition, we have measured the effect of pentobarbital on the desensitization rates of both receptors over the same wide range of pentobarbital concentration. The effects on desensitization suggest how the measurements of equilibrium binding of GABA to membrane preparations are correlated with the measurements of functional responses of the GABA receptor, although these affinities are more than 100-fold greater than those mediating the functional responses. Additionally, a new conformational change, besides channel opening and desensitization, was revealed by the recovery of activity of the slower desensitizing receptor, following its very rapid inhibition in high concentrations of pentobarbital.

Materials and Methods

A membrane preparation was prepared from the cerebral cortex of male Sprague-Dawley rats, 4–6 weeks old, as described previously (Cash & Subbarao, 1987c). Instead of the Ficoll gradient, the pellet was resuspended in 10 mL of solution B [145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, 10 mM *N*-2-(hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7.5], diluted to 30 mL, and centrifuged for 30 min, 23640*g*. The final pellet was resuspended in 10 mL of solution B and adjusted to 750 μg of protein/mL. The pentobarbital used was the commercially available sodium salt of the racemic mixture.

GABA-Mediated Chloride Ion Exchange. The GABA-mediated $^{36}Cl^-$ influx into the membrane vesicles was measured with the quench–flow apparatus (Cash & Hess, 1981) as described previously (Cash & Subbarao, 1987d). The measurements were made in solution B at 30 °C, pH 7.5. The receptor channels were opened on mixing the membrane preparation with a solution containing GABA and $^{36}Cl^-$ and closed on mixing the suspension with a solution to give 1000 μM bicuculline methiodide. The pentobarbital was added in the GABA solution. In some cases a short preincubation with pentobarbital was carried out by using an additional mixing stage in the quench–flow machine before the admixture with the GABA solution.

Unspecific $^{36}Cl^-$ influx, background, measured in the same way in the absence of GABA, was subtracted from the total influx to give the GABA-mediated, specific $^{36}Cl^-$ influx. All determinations of specific and unspecific chloride influx were made in triplicate.

Activation and Desensitization of the Receptor. The activity of the receptor was followed in solution B, 30 °C, pH 7.5, by using a quench–flow technique as previously described for desensitization (Cash & Subbarao, 1987c; Aoshima et al., 1981). After incubation with pentobarbital or pentobarbital and GABA, the GABA-mediated influx of $^{36}Cl^-$ in a constant assay time was measured in a second incubation as described above. All determinations were made in triplicate. When the pentobarbital concentration in the preincubation was varied in an experiment, pentobarbital as well as GABA was added with the $^{36}Cl^-$ to keep the composition of the chloride-influx assay solution the same for the different preincubations. To compensate for the inhibition due to pentobarbital, the duration of the assay incubation was sometimes increased from 320 to 2000 ms.

Treatment of Data. The previous description of ion exchange mediated by two types of receptor, which undergo desensitization, on the same population of membrane vesicles (Cash & Subbarao, 1987c) is extended to include the case in which one of the receptors is activated during the measurements. The receptor-mediated influx of $^{36}Cl^-$ into the vesicles is described by

$$\frac{M_t}{M_\infty} = 1 - \exp\left\{-\left[J_A\left(\frac{1-\exp(-\alpha t)}{\alpha}\right) + J_{Bf}\left(\frac{1-\exp(-\beta t)}{\beta}\right) - (J_{Bf} - J_{Bi})\left(\frac{1-\exp[-(\beta+k)t]}{\beta+k}\right)\right]\right\} \quad (1)$$

where M_t/M_∞ is the fractional equilibration of $^{36}Cl^-$ that occurs in the incubation time t and J_A and J_{Bi} are the initial first-order rate constants for $^{36}Cl^-$ influx mediated by the faster and slower desensitizing receptors, respectively. In the presence of pentobarbital the slower desensitizing receptor is converted, with a rate constant k, to a more active (less inhibited by pentobarbital) form characterized by a higher $^{36}Cl^-$ influx rate constant J_{Bf}. The faster desensitizing and the slower desensitizing receptors are desensitized with rate constants α and β, respectively. If $k << \beta$, eq 1 simplifies to the previous eq 1 (Cash & Subbarao, 1987c) where $J_{Bi} = J_B$, and if $k >> J_{Bf} - J_{Bi}$, it simplifies to the previous eq 1 where $J_{Bf} = J_B$.

If the receptors are preincubated with GABA or pentobarbital, so that desensitization or activation of the receptor proceeds before the ion-influx assay, the values of the ion-flux rate constants are altered according to eq 2–4. The subscript

$$J_A = J_{A(t_p=0)} \exp(-\alpha_p t_p) \quad (2)$$

$$J_{Bf} = J_{Bf(t_p=0)} \exp(-\beta_p t_p) \quad (3)$$

$$J_{Bi} = [J_{Bi(t_p=0)} + (J_{Bf(t_p=0)} - J_{Bi(t_p=0)})[1 - \exp(-k_p t_p)]] \exp(-\beta_p t_p) \quad (4)$$

p denotes that the values of the constants are those in the preincubation. Equations 1–4 give rise to eq 5, which describes

$$-\ln(1 - M_t/M_\infty) = A\exp(-\alpha_p t_p) + D\exp(-\beta_p t_p) - C\exp[-(\beta_p + k_p)t_p] \quad (5)$$

the change in $^{36}Cl^-$ influx, in a constant assay time, due to a prior incubation with GABA and/or pentobarbital. The activity changes in three phases with amplitudes given by A, D, and C. When the assay (second) incubation has constant conditions and duration, A, D, and C are complex constants given by eq 6–8.

$$A = J_{A(t_p=0)}\left(\frac{1-\exp(-\alpha t)}{\alpha}\right) \quad (6)$$

$$D = J_{Bf(t_p=0)}\left[\left(\frac{1-\exp(-\beta t)}{\beta}\right) - \left(\frac{1-\exp[-(B+k)t]}{\beta+k}\right)\right] + J_{Bf_p(t_p=0)}\left(\frac{1-\exp[-(B+k)t]}{\beta+k}\right) \quad (7)$$

$$C = (J_{Bf_p(t_p=0)} - J_{Bi(t_p=0)})\left(\frac{1 - \exp[-(B + k)t]}{\beta + k}\right) \quad (8)$$

The time t_p is the duration of the preincubation and t is the duration of the $^{36}Cl^-$-influx assay in the second incubation. The subscript $t_p = 0$ denotes that the value is at zero preincubation time. If the conditions in the two incubations are the same, so that the final equilibrium position of activation does not vary due to a change in solution composition, D is equal to B in the previous eq 4 (Cash & Subbarao, 1987c). If $k_p >> \beta_p$, eq 5 simplifies to the previous eq 4, where the amplitude term B is D corresponding to J_{Bf}. If $k_p << \beta_p$, eq 5 simplifies to the previous eq 4 where B is $(D - C)$ corresponding to J_{Bi}.

For the measurements of isotope exchange after preincubation of the receptor (t_p is varied; t is constant), eq 5 was fitted to the experimental data (e.g., Figures 2 and 3A). For the measurements of the time course of $^{36}Cl^-$ influx (t is varied; t_p is constant, usually zero), eq 1 was fitted to the experimental data (e.g., Figures 1 and 3B). Where necessary, the values of α, β, and k determined in preincubation experiments in the same conditions were used in this curve fitting. In practice, activation of the receptor was not detected much below ca. 1000 μM pentobarbital where $J_{Bi} \rightarrow J_{Bf}$, and so eq 1 used for the influx time course experiments became equivalent to the previous eq 1 (Cash & Subbarao, 1987c) and eq 5 used for the preincubation experiments became equivalent to the previous eq 4, at low pentobarbital concentrations.

Results

The GABA-mediated $^{36}Cl^-$ influx was followed in the presence of various concentrations of pentobarbital at different GABA concentrations. The ion exchange proceeded in two phases (Figures 1 and 3) previously shown to be due to two receptors distinguishable on the basis of their desensitization rates (Cash & Subbarao, 1987c,d). In a different type of experiment the desensitization (and activation) processes were followed by measuring the change in $^{36}Cl^-$ influx in constant assay conditions after different times of preincubation of the membrane with GABA and/or pentobarbital (Cash & Subbarao, 1987c). The activity was seen to decrease in two phases (Figure 2). In a special case when the preincubation and assay incubation each contained high concentrations of pentobarbital, an *increase* of activity during the preincubation was seen with a rate that became much faster than that of the slower desensitization.

With low (significantly less than saturating) concentrations of GABA and low (<200 μM) concentrations of pentobarbital, the progress of $^{36}Cl^-$ influx in the slower phase was accelerated and increased in amplitude (counts at the final level) with increasing pentobarbital concentration (Figure 1A,B). Analysis of the shapes of the ion-influx curves showed that not only the chloride-exchange rate (J_B) but also the rate of desensitization (β) was increased by the pentobarbital (Figures 4 and 5). The increase in amplitude (extent of chloride influx) occurred because the ion-flux rate was increased more than the desensitization rate (Cash & Subbarao, 1987d; Cash & Hess, 1984). With 10 μM GABA, the ratio J_B/β increased from 0.55 with no pentobarbital to 3.0 with 200 μM pentobarbital. The increase in β was confirmed by the direct measurements of desensitization rate measured in the preincubation experiments (Figure 2). The increases in both J_B and β became significant at approximately the same pentobarbital concentration, above ca. 30 μM pentobarbital with 20 μM GABA (Figures 4B and 5B). In contrast, the amplitude of the $^{36}Cl^-$ influx in the fast phase was increased only slightly. The ratio J_A/α of 0.43 (10 μM GABA) was increased

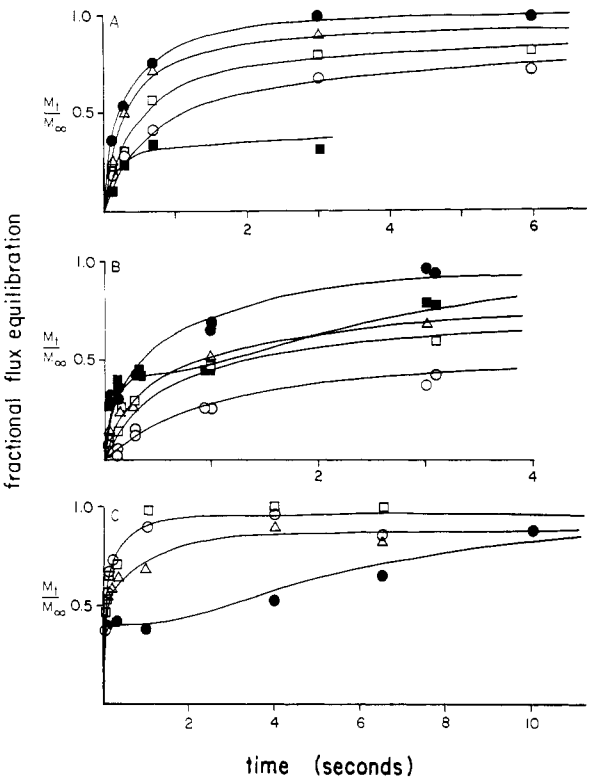


FIGURE 1: Effect of pentobarbital on the time course of GABA-mediated $^{36}Cl^-$ exchange into the membrane vesicles, with different concentrations of GABA, measured as described under Materials and Methods (Cash & Subbarao, 1987d). This illustrates the immediate acceleration of both phases by pentobarbital with less than saturating GABA and the immediate inhibition and subsequent slower reactivation of the second phase by high pentobarbital concentrations at all GABA concentrations. The membrane suspension (225 μL, at a concentration of 750 μg of protein/mL) was mixed with an equal volume of solution B containing GABA and $^{36}Cl^-$ (15 μCi/mL). The GABA-mediated chloride exchange was terminated by mixing with the same volume of solution B containing bicuculline methiodide (3000 μM). The GABA-mediated chloride exchange gave an uptake maximally of ca. 2000 counts (10-min count) on a nonspecific background increasing from 750 to 2000 counts after a 3-s incubation. The points are the means of three determinations. The reproducibility of the determinations was within ±5% (standard deviation) of the total specific measurement. The lines are calculated from eq 1 with the values of the parameters indicated. The chloride-exchange activities of the two receptors are given by the first-order ion-flux rate constants J_A and J_B, which are depleted with desensitization rates given by the first-order rate constants α and β, respectively, in the two phases. Except where otherwise stated, the inhibition by pentobarbital and the corresponding reactivation are too small to be measurable ($J_B = J_{Bi} = J_{Bf}$). The $^{36}Cl^-$ inside the vesicles, M_t, is expressed as a fraction of that at equilibration of chloride exchange, M_∞, determined with saturating GABA (1000 μM) and 6-s incubation time. (A) With low (20 μM) GABA concentration and (○) no pentobarbital (J_A = 1.1 s^{-1}, α = 1.5 s^{-1}, J_B = 0.16 s^{-1}, β = 0.1 s^{-1}), (□) 20 μM pentobarbital (J_A = 1.6 s^{-1}, α = 1.6 s^{-1}, J_B = 0.20 s^{-1}, β = 0.12 s^{-1}), (△) 60 μM pentobarbital (J_A = 2.5 s^{-1}, α = 2.2 s^{-1}, J_B = 0.50 s^{-1}, β = 0.25 s^{-1}), (●) 500 μM pentobarbital (J_A = 3.0 s^{-1}, α = 6 s^{-1}, J_B = 1.5 s^{-1}, β = 0.3 s^{-1}), and (■) 3000 μM pentobarbital (J_A = 2.1 s^{-1}, α = 6.0 s^{-1}, J_B < 0.05 s^{-1}). (B) With low (10 μM) GABA concentration and (○) no pentobarbital (J_A = 0.45 s^{-1}, α = 1.0 s^{-1}, J_B = 0.055 s^{-1}, β = 0.1 s^{-1}), (□) 50 μM pentobarbital (J_A = 1.0 s^{-1}, α = 2.0 s^{-1}, J_B = 0.2 s^{-1}, β = 0.15 s^{-1}), (△) 100 μM pentobarbital (J_A = 1.5 s^{-1}, α = 3.0 s^{-1}, J_B = 0.35 s^{-1}, β = 0.3 s^{-1}), (●) 400 μM pentobarbital (J_A = 3.0 s^{-1}, α = 7.0 s^{-1}, J_B = 1.0 s^{-1}, β = 0.3 s^{-1}), and (■) 1000 μM pentobarbital (J_A = 5.3 s^{-1}, α = 9.5 s^{-1}, J_{Bf} = 1.0 s^{-1}, β = 0.1 s^{-1}, J_{Bi} = 0, k = 0.3 s^{-1}). The duplicate points refer to two different experiments, with similar preparations. Each point is the average of triplicate determinations. (C) With high (500 μM) GABA concentration and (○) no pentobarbital (J_A = 13 s^{-1}, α = 18 s^{-1}, J_B = 2.4 s^{-1}, β = 1.0 s^{-1}), (□) 200 μM pentobarbital (J_A = 14 s^{-1}, α = 20 s^{-1}, J_B = 2.8 s^{-1}, β = 1.3 s^{-1}), (△) 400 μM pentobarbital (J_A = 14 s^{-1}, α = 20 s^{-1}, J_{Bi} = 0.8 s^{-1}, β = 0.7 s^{-1}), and (●) 1000 μM pentobarbital (J_A = 10 s^{-1}, α = 20 s^{-1}, J_{Bi} = 0, J_{Bf} = 1.0 s^{-1}, β = 0.1 s^{-1}, k = 0.06 s^{-1}).

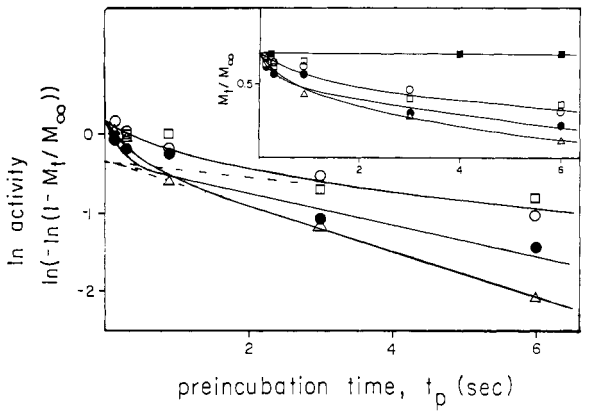


FIGURE 2: Effect of pentobarbital on the desensitization of GABA receptor measured by following the loss of chloride-exchange activity after progressive times of prior incubation with GABA, as described under Materials and Methods (Cash & Subbarao, 1987c). The membrane suspension (225 μL, at a concentration of 750 μg of protein/mL) was mixed with an equal volume of solution B containing GABA. After a predetermined preincubation time, the suspension was mixed with solution B containing GABA (225 μL) and the $^{36}Cl^-$ influx was measured in a second incubation as described in the legend to Figure 1. The GABA concentration is 20 μM. The points are means of three determinations, the precision of which is within ±5% of the equilibrium measurement. This is a semilog (first-order) plot of the data showing the biphasic desensitization. (Inset) Variation of $^{36}Cl^-$ influx as a function of preincubation time with pentobarbital. The initial activity, at the ordinate intercept, is given by experiments with no GABA in the preincubation. The $^{36}Cl^-$ inside the vesicles, M_t, is expressed as a fraction of that at equilibration of chloride exchange, M_∞, determined in a time of 6 s with no GABA or pentobarbital present in the preincubation. The $^{36}Cl^-$ influx incubation lasts for 320 ms in the presence of 500 μM GABA and 667 μM pentobarbital. Measurements made with no GABA or pentobarbital in the preincubation (■) are independent of t_p and represent zero preincubation time and no prior desensitization. The lines are calculated from eq 5, which for these examples is approximated to the previous eq 4 (Cash & Subbarao, 1987c), with the values of the parameters indicated. The first-order rate constants for desensitization of the two phases of chloride exchange are α and β, respectively. The preincubation contains 20 μM GABA with (○) no pentobarbital (α = 1.2 s^{-1}, β = 0.11 s^{-1}), (□) 20 μM pentobarbital (α = 1.3 s^{-1}, β = 0.11 s^{-1}), (△) 100 μM pentobarbital (α = 2.9 s^{-1}, β = 0.3 s^{-1}), and (●) 1000 μM pentobarbital (α = 5 s^{-1}, β = 0.2 s^{-1}).

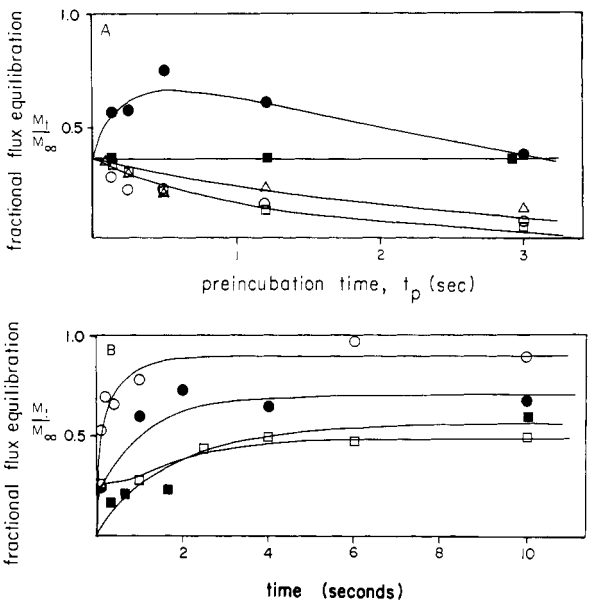


FIGURE 3: Experiments demonstrating the reactivation of the receptor after its relatively very rapid inhibition in the presence of pentobarbital. The experimental procedures are as described in the legends to Figures 1 and 2. The rate constants J_A, J_B, α, and β are as defined in the legend to Figure 1. The rate constant for reactivation of the receptor in the preincubation is k_p. (A) $^{36}Cl^-$ influx measured in an assay incubation (320 ms, containing 1000 μM GABA and 2000 μM pentobarbital) following preincubation with buffer solution B containing (■) no GABA or pentobarbital, (○) 500 μM GABA and no pentobarbital (β = 0.95 ± 0.3 s^{-1}), (□) 500 μM GABA and 200 μM pentobarbital (β = 1.1 ± 0.3 s^{-1}), (△) 500 μM GABA and 1000 μM pentobarbital (β = 0.6 ± 0.2 s^{-1}, k_p = 0.1 s^{-1}), and (●) 500 μM GABA and 3000 μM pentobarbital (β = 0.39 ± 0.06 s^{-1}, k_p = 3.5 s^{-1}). The values for the rate constants given correspond to the lines that are calculated from eq 5. With the largest pentobarbital concentration (3000 μM) in the preincubation, the rate and extent of the recovery of activity is clearly measurable in the second incubation. The fraction of $^{36}Cl^-$ exchange, M_t/M_∞, was determined as described in the legend to Figure 2. (B) Time course of the $^{36}Cl^-$ influx measured after preincubation with or without pentobarbital. Chloride exchange measured with 1000 μM GABA and (○) no pentobarbital (J_A = 9 s^{-1}, α = 22 s^{-1}, J_B = 2.6 s^{-1}, β = 1.3 s^{-1}), (□) 1333 μM pentobarbital (J_A = 6.5 s^{-1}, α = 22 s^{-1}, J_{Bi} = 0, J_{Bf} = 0.9 s^{-1}, β = 0.7 s^{-1}, k = 0.3 s^{-1}), (●) 1333 μM pentobarbital following a preincubation for 500 ms with 2000 μM pentobarbital (J_A = 4 s^{-1}, α = 22 s^{-1}, J_{Bf} = 0.9 s^{-1}, J_{Bi} = 0.7 s^{-1}, β = 0.7 s^{-1}; this corresponds to a reactivation rate constant k_p = 3 s^{-1} in the preincubation), and (■) 1333 μM pentobarbital following preincubation for 3000 ms with 2000 μM pentobarbital (J_A negligible, J_B = 0.6 s^{-1}, β = 0.7 s^{-1}). The values for the rate constants given correspond to the lines, which are calculated from eq 1. The inhibition by 1333 μM pentobarbital (□ compared with ○) is seen, as well as activation after preincubation with 2000 μM pentobarbital (● compared with □) and activation during the assay (sigmoid shape of □).

by only ca. 15% by 200 μM pentobarbital. However, the preincubation experiments measuring desensitization rate (Figure 2) and analysis of the $^{36}Cl^-$ influx curves (Figure 1A,B) showed that both chloride exchange rate and receptor desensitization rate were accelerated by approximately the same factor of 4 (Figures 4 and 5).

With high (near-saturating) GABA concentrations (500–1000 μM), the $^{36}Cl^-$ influx was not increased (Figure 1C) in this range of relatively low pentobarbital concentration. While the rates of chloride exchange (J_A and J_B) were increased only marginally, the rates of desensitization (α and β) were not changed significantly (Figure 6). This was confirmed by the direct measurements of desensitization (Figure 3A).

With higher concentrations of pentobarbital (>500 μM) an inhibitory effect on the course of $^{36}Cl^-$ influx was observed, which again distinguished between the two phases. With low concentrations of GABA (e.g., 10 or 20 μM) and above 1000 μM pentobarbital (Figure 1A,B) the second (slower) phase was completely inhibited in a time range of up to ca. 1 s, while the faster phase was not much changed. On a longer time scale the rate of the second phase of ion flux increased during the measurement, giving rise to a sigmoid $^{36}Cl^-$-influx curve (Figure 1B). The preincubation experiments (Figure 2) as well as the analysis of the chloride-influx curves showed that the desensitization rate β of the slower desensitizing receptor was decreased with increasing pentobarbital above 400 μM pentobarbital (Figure 4B). In contrast, the desensitization rate α of the faster desensitizing receptor continued to increase with increasing pentobarbital concentration up to at least 1000 μM (Figure 4A). Similarly, the initial rate constant for chloride exchange, J_B, for the slower desensitizing receptor is decreased sharply with increasing pentobarbital (Figure 5B). In contrast, the initial chloride-influx rate constant, J_A, obtained from the fast phase of ion flux is not decreased in these pentobarbital concentrations (Figure 5A), although if the measurements are compared with the extrapolated activation curve, some inhibition is apparent.

With high (saturating) GABA concentrations (500 or 1000 μM) a similar inhibition of the second phase of $^{36}Cl^-$ influx is seen (Figures 1C and 3B) above 200 μM pentobarbital,

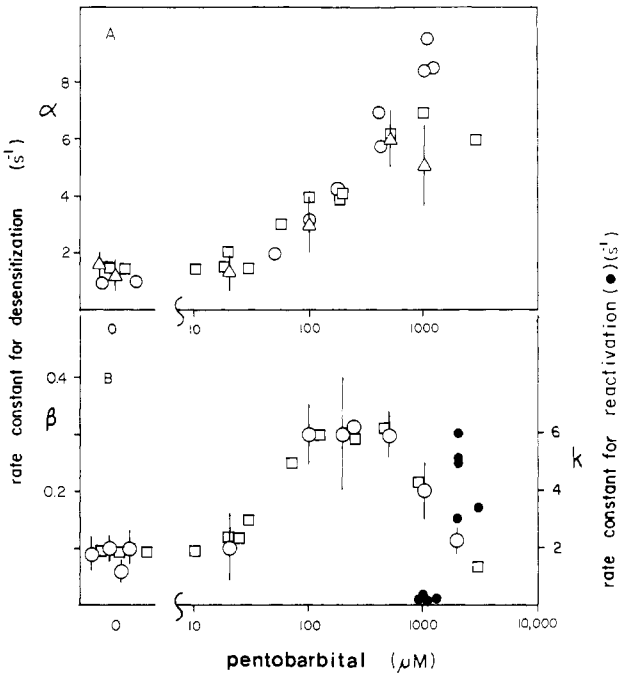


FIGURE 4: Dependence of the rate of desensitization with low GABA concentration on the concentration of pentobarbital, for the faster and the slower desensitizing receptor. The parameters are defined in the legend to Figure 1. (A) The faster desensitizing receptor. Variation of α with increasing pentobarbital concentration: (△) measured with a low (20 μM) GABA concentration in the preincubation in experiments measuring $^{36}Cl^-$ influx in a constant assay following progressive preincubation times (e.g., Figure 3); (□) measured with 20 μM GABA in experiments following the time course of $^{36}Cl^-$ influx (e.g., Figure 1); (○) measured with 10 μM GABA in experiments following the time course of $^{36}Cl^-$ influx. (B) The slower desensitizing receptor. Variation of β with increasing pentobarbital concentration: (○) measured with a low (20 μM) GABA concentration in the preincubation experiments as above; (□) measured with 20 μM GABA in experiments following the time course of chloride exchange as above. The filled circles (●) show the estimated rates of reactivation of the receptor, which had been rapidly inhibited in high concentrations of pentobarbital. These rates of activation were independent of GABA concentration (see Results).

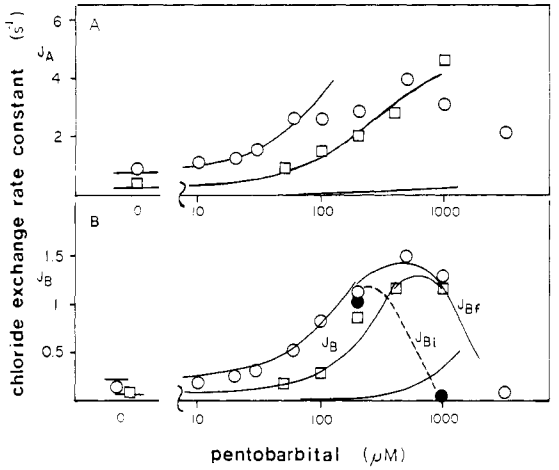


FIGURE 5: Dependence of the rate of chloride exchange with low GABA concentration on the concentration of pentobarbital for the faster and the slower desensitizing receptors. The parameters are defined in the legend to Figure 1. (A) Faster desensitizing receptor. Variation of the initial rate constant J_A with (□) 10 μM and (○) 20 μM GABA, determined from the time course of $^{36}Cl^-$ influx (e.g., Figure 1). The lines are calculated with eq 9 and the values of the parameters in Table I. The lowest line gives the influx rate constant predicted in the presence of 1 μM GABA. (B) Slower desensitizing receptor. Variation of J_B with (□) 10 μM and (○) 20 μM GABA, determined from the time course of chloride influx (e.g., Figure 1). The lines at low pentobarbital concentration are calculated with eq 9 and the values of the parameters in Table I. The lowest line gives the rate constant predicted for the presence of 1 μM GABA. The lines at higher pentobarbital concentrations, in which inhibition by pentobarbital is significant, describe the value immediately after pentobarbital addition (●), J_{Bi} (dashed line), and the value after the receptor has relaxed at that concentration of pentobarbital (○, □), J_{Bf} (continuous line).

reaching complete inhibition initially by 1000 μM. This inhibition by pentobarbital again dramatically illustrates the dichotomy of the chloride influx into the two phases mediated by the two receptors. Sigmoid-shaped $^{36}Cl^-$-influx curves, of the second phase, are again in evidence when the inhibition is large. The preincubation experiments (Figure 3A) as well as analysis of the $^{36}Cl^-$-influx curves show that the desensitization rate β is decreased above 1000 μM pentobarbital (Figure 6B). In contrast, the desensitization rate α of the faster desensitizing receptor remains constant up to at least 1000 μM pentobarbital (Figure 6A). Similarly, the chloride-flux rate constant in the second phase of ion flux, J_B, is decreased with increasing pentobarbital (Figure 6B) above 200 μM pentobarbital. In contrast the chloride-flux rate in the fast phase, J_A, is decreased only slightly at 1000 μM pentobarbital (Figure 6A) by a much smaller extent than J_B.

With low pentobarbital concentrations, where inhibition by pentobarbital was not detected, the $^{36}Cl^-$-influx curves were fitted to a simplified form of eq 1, where $J_{Bi} = J_{Bf} = J_B$, which is equivalent to the previous eq 1 (Cash & Subbarao, 1987c). At higher pentobarbital concentrations where inhibition was detectable, eq 1 where $J_{Bi} < J_{Bf}$ was used for the following reasons. (1) The calculated lines ($J_{Bi} = J_{Bf}$) fit the points lees well than expected ($\chi^2 > 1$); in extreme cases the experimental points appeared to follow a sigmoid curve. (2) The apparent value of β to fit the influx curves (with $J_{Bi} = J_{Bf}$) was significantly less than that measured in the preincubation experiments. The observed behavior, described by eq 1, is consistent with an initial flux rate constant J_{Bi} characterizing a receptor that was relatively very rapidly inhibited by pentobarbital, which is converted during the measurement to a larger, final ion-flux rate constant J_{Bf} characterizing a receptor that is less inhibited by pentobarbital.

In the preincubation experiments, devised to measure the course of desensitization in the first incubation, when pentobarbital was present in the first incubation, it was consequently present in the second (assay) incubation. With high pentobarbital concentrations (above ca. 1000 μM pentobarbital in the assay) the amplitude of the $^{36}Cl^-$ influx assay was reduced. In some experiments the assay time was increased to compensate for this. In conditions where the assay amplitude was decreased by pentobarbital and a high pentobarbital concentration (>1000 μM) was present in the preincubation, an *activation* of the receptor activity during the preincubation was seen. This is illustrated by an experiment with high pentobarbital concentrations in both incubations, devised to enhance the observation of reactivation (Figure 3A). In several experiments, with 2000 or 3000 μM pentobarbital in the preincubation and 1333 μM pentobarbital in the assay solution, with high or low GABA concentrations, the $^{36}Cl^-$ influx in the second incubation passed through a maximum measured with varying preincubation time at ca. 200–500-ms preincubation, giving a ca. 50% increase in $^{36}Cl^-$ influx. The reactivation rate constant k_p could be determined from fitting eq 5 to the results, since k was much larger than β and a fast phase of desensitization was absent in some preparations (Cash & Subbarao, 1987a,c). The value of k_p was not significantly dependent on GABA concentration but increased steeply with pentobarbital concentration (Figure 4B). With 1000 μM pentobarbital in

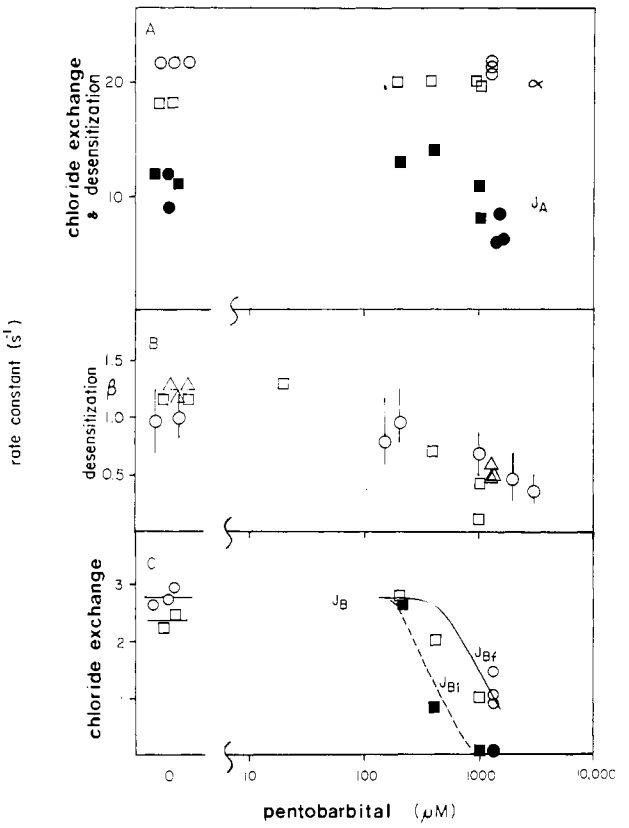


FIGURE 6: Dependence of chloride-exchange rate and desensitization rate with high GABA concentration on the concentration of pentobarbital for both phases of ion flux due to the two receptors. The parameters are defined in the legend to Figure 1. (A) Faster desensitizing receptor. Variation of α (open symbols) and J_A (filled symbols) measured with 500 μM (□, ■) and 1000 μM (○, ●) GABA with increasing pentobarbital concentration. (B) Slower desensitizing receptor. Variation of the desensitization rate constant β (○) measured with 500 μM GABA in the preincubation in experiments measuring $^{36}Cl^-$ exchange in a constant assay following progressive preincubation times (e.g., Figure 3) and measured with (□) 500 μM and (△) 1000 μM GABA in experiments following the time course of $^{36}Cl^-$ influx (e.g., Figure 1). (C) Slower desensitizing receptor. Variation of J_B measured with 500 μM (□, ■) and 1000 μM (○, ●) GABA with increasing pentobarbital concentration. At high pentobarbital concentrations, where inhibition is significant, the filled symbols and the dashed line give the values J_{Bi} immediately after the addition of pentobarbital; the open symbols and continuous line give the values after the receptor has relaxed at that pentobarbital concentration J_{Bf}.

several experiments $k_p \lesssim 0.1$ s^{-1}. With 500 μM GABA, measured values in different experiments were $k_p = 5$ s^{-1} (2000 μM pentobarbital) and $k_p = 3.5$ s^{-1} (3000 μM pentobarbital). With 20 μM GABA, $k_p = 6$ s^{-1} (2000 μM pentobarbital) was measured. This activation of chloride influx did not require the presence of added GABA in the preincubation and was observed with pentobarbital alone above 600 μM. With 2000 μM pentobarbital in the preincubation for 300 ms, a 50% increase in chloride influx ($k_p = 6$ s^{-1}) was seen in the assay with 1333 μM pentobarbital and 1000 μM GABA. This direct observation of activation of the inhibited receptor by pentobarbital is consistent with the sigmoid $^{36}Cl^-$-influx curves observed. To support this interpretation, the time course of the chloride exchange was followed after preincubation of the membrane with pentobarbital. In the experiment shown (Figure 3B) the conditions of $^{36}Cl^-$ influx are those of a typical assay incubation. The inhibition of $^{36}Cl^-$ influx by 1333 μM pentobarbital is seen. The sigmoid shape of the second-phase influx curve reflects reactivation of the receptor and the occurrence of some $^{36}Cl^-$ influx, before this is reduced to a negligible rate due to receptor desensitization. After exposure to 2000 μM pentobarbital for 500 ms, the $^{36}Cl^-$ influx under the same conditions follows a steeper curve due to partially reactivated receptor. The initial $^{36}Cl^-$-influx rate was increased from a negligible rate to ca. $^1/_2$ the rate in the absence of pentobarbital (see Figure 6B). With a longer preincubation time of 3 s the amplitude of $^{36}Cl^-$ influx was again reduced by desensitization of the receptor, as seen in the usual preincubation experiment with constant assay time (Figure 3A). These low rates of desensitization are consistent with those measured directly by the preincubation method, due to pentobarbital at high concentrations with no added GABA (see below). From the accelerated chloride-influx curves after preincubation with pentobarbital, values for the reactivation rate constant in the preincubation, k_p, were calculated. With 1000 μM GABA, $k_p = 0.05$ s^{-1} (1000 μM pentobarbital) and $k_p = 3.0$ s^{-1} (2000 μM pentobarbital). The reactivation rate constant k was also estimated by fitting eq 5 to the sigmoid influx curves. In this curve fitting the first phase of ion flux is well separated in time, $J_{Bi} \simeq 0$, and β and J_{Bf} are known from different experiments in the same conditions. With 1000 μM pentobarbital, estimated values were $k = 0.3$ s^{-1}, 0.06 s^{-1}, and 0.05 s^{-1} with 10, 500, and 1000 μM GABA, respectively. With 1333 μM pentobarbital, values of $k = 0.25$ s^{-1} and 0.10 s^{-1} were measured with 1000 μM GABA. These values, indicating a very steep dependence of reactivation rate on pentobarbital concentration (Figure 4B), were supported by the determinations from the influx curves after preincubation with pentobarbital and also by the experiments measuring the time course of activation and desensitization.

In experiments with pentobarbital alone, with no added GABA, desensitization measured by the preincubation technique was undetectable with 200 μM pentobarbital in the times of these experiments. Even with higher concentrations of pentobarbital no fast phase of desensitization was detected, but with 600 μM pentobarbital a little desensitization, $\beta \lesssim 0.1$ s^{-1} was seen and with 2000 μM pentobarbital $\beta \lesssim 0.2$ s^{-1}. The presence of a very low concentration of endogenous GABA in solution, active in the presence of pentobarbital but not detected alone (Cash & Subbarao, 1987d), cannot be ruled out. In measurements of specific $^{36}Cl^-$ influx with pentobarbital alone, no fast phase was detected, but a slow phase giving, at most, $M_t/M_\infty = 0.4$ in 6.5 s was observed, which corresponded to $J_B \lesssim 0.14$ s^{-1}.

Discussion

The channel-opening equilibria and the receptor-desensitization rates of both receptors, mediated by less than saturating GABA concentrations, were increased with increasing pentobarbital up to a concentration of ca. 200 μM pentobarbital (Figures 4 and 5). This gave an increase in final $^{36}Cl^-$ influx (in times longer than 2–3 s) only with the slower desensitizing receptor, for which chloride exchange was enhanced more than desensitization. This is because the final level of ion influx after desensitization depends on the ratio of the chloride-exchange rate and the desensitization rate, J_A/α or J_B/β (Cash & Hess, 1984), and this ratio was not much changed for the faster desensitizing receptor over a 4-fold acceleration of chloride-exchange rate J_A and desensitization rate α. When the receptors were saturated with GABA, enhancement of desensitization due to pentobarbital was negligible and the increase in chloride exchange was very small for each receptor (Figure 6). This shift of the response curve to lower GABA concentrations, for both chloride-influx rate (channel-opening equilibrium) and desensitization rate, for both receptors is explained by an apparent enhancement of GABA binding due to pentobarbital. Pentobarbital is acting

as an activating, allosteric effector of the GABA receptor (Hammes & Wu, 1974; Monod et al., 1965).

GABA-induced responses in the brain, due to chloride channels, are enhanced and prolonged by pentobarbital (e.g., Nicoll, 1975; Evans, 1979; Connors, 1981; Higashi & Nishi, 1982; Alger & Nicoll, 1982; Simmonds, 1981, Lodge & Curtis, 1978; Nicoll & Wojtowicz, 1980; Akaike et al., 1985; MacDonald & Barker, 1979; Schultz & MacDonald, 1981; Ransom & Barker, 1976; Barker & Ransom, 1978; Parker et al., 1986). This enhancement of chloride-channel opening occurs in the concentration region 50–600 μM, which pharmacological experiments showed corresponds to anesthetic effects (Richards, 1972). This concentration range compares with that of the enhancement of GABA-mediated chloride ion exchange (Figures 4–6). It was suggested that the enhancement of channel opening is due to binding of pentobarbital at allosteric effector sites (MacDonald & Barker, 1979). This would be consistent with an increase in the mean open-channel lifetime (Study & Barker, 1981; Simmonds, 1981; Mathers, 1985; Parker et al., 1986). It is supported by the observations that pentobarbital causes a shift of the response/[GABA] curve to lower GABA concentrations with little increase in maximum response (Barker & Ransom, 1978; Parker et al., 1986; Akaike et al., 1984).

During the course of this work, reports appeared describing the enhancement by pentobarbital of chloride ion exchange, with membrane preparations containing vesicles, mediated by GABA (Allan & Harris, 1986) or muscimol (Schwartz et al., 1986b). This was observed in the same region of pentobarbital concentration as those discussed above. The response/[muscimol] curve was shifted to lower concentrations of muscimol, similarly to our results with GABA and the electrophysiological results.

In the absence of added GABA, pentobarbital at higher concentrations was found to stimulate transmembrane chloride exchange with brain slices (Wong et al., 1984) and a membrane preparation (Schwartz et al., 1984, 1985, 1986a,b). The inhibitory effect of GABA receptor antagonists on the background chloride exchange (no added GABA) indicated the presence of some endogenous GABA in solution in these preparations (Schwartz et al., 1984, 1985, 1986a,b), the effect of which might be enhanced by the added pentobarbital. However, some indications have been reported, from electrophysiological experiments, that pentobarbital may act on a different receptor in the absence of GABA (e.g., Huang & Barker, 1980; Mathews & Barker, 1980; Higashi & Nishi, 1982; Nicoll & Wojtowicz, 1980; Parker et al., 1986). Since different GABA receptors exist that respond in different time ranges depending on the rates of desensitization due to GABA (Cash & Subbarao, 1987c,d), with the predominant receptor becoming desensitized more rapidly, it is possible that a different GABA receptor could be observed, in very short times or in the absence of GABA than in longer times, when the predominant, faster desensitized activity has been removed.

In approximately the same concentration range that increased the electrophysiological response and chloride conductance or transmembrane $^{36}Cl^-$ isotope exchange mediated by the GABA receptor, pentobarbital increased the binding of submicromolar concentrations of GABA or muscimol to membrane prepared from brain in equilibrium binding measurements (Skerritt et al., 1983; Olsen & Snowman, 1982; Whittle & Turner, 1982; Quast & Brenner, 1983; Thyagarajan et al., 1983; Willow & Johnston, 1981a,b). Although there have been some differences concerning whether the number of binding sites or the affinity is increased, the observation of changed dissociation rates of GABA (Willow & Johnston, 1981c) or muscimol (Olsen & Snowman, 1982) by pentobarbital indicated that the sites may be altered. However, the affinities for GABA of the different types of site involved in these measurements, K_D = 5 nM, 250 nM, and probably $\sim$1 μM, are much greater than those of the binding processes causing channel opening or desensitization, K_D = 150 μM (Cash & Subbarao, 1987d), as measured in electrophysiological or ion-flux experiments. If the sites measured in the equilibrium binding assay are the sites involved in the functional responses of the receptor, they have been changed by the conformational changes, such as desensitization, that result from exposure to GABA during the binding assay for a prolonged period, sometimes as long as 15 min. The similarity of the pentobarbital concentrations causing increased high-affinity binding and increased desensitization rate (Figures 4 and 6) suggests that the enhanced (submicromolar) binding reflects a concomitant enhanced equilibrium in favor of the (higher affinity) desensitized state.

It has been suggested that a mechanism proposed for allosteric regulatory enzymes (Monod et al., 1965) can explain the receptor-mediated changes in the electrical properties of biological membranes (Changeux et al., 1967; Karlin, 1967; Changeux & Podleski, 1968; Edelstein, 1972). The change in membrane permeability to specific ions was related to a change in conformation of an oligomeric receptor complex, although the exact nature of the relationship was uncertain. The study of the fluctuations of small electrophysiological signals (Katz & Miledi, 1970, 1971; Anderson & Stevens, 1977; Neher & Stevens, 1977) and the introduction of a patch clamp technique enabling the observation of ionic conduction through a single channel (Neher & Sakmann, 1976), besides the specificity of the channels, indicated that the postsynaptic signal results from the *independent* opening of many small channels with the size of a small molecule. The identification of the conformational change of a single receptor complex with the opening of a single channel (e.g., Magleby & Stevens, 1972; Katz & Miledi, 1972; Adams, 1975; DelCastillo & Katz, 1957) gives a direct relationship between the membrane permeability to a specific ion and the fraction of the receptor in the open-channel state. The channel-opening event can be considered as a *concerted* conformational change (Adams, 1975; Cash & Hess, 1980) in the protein complex, itself giving rise to the cooperativity of response (Cash & Hess, 1980). The cooperativity of ligand binding observed in direct measurements of ligand binding, which are performed in longer times, results from additional conformation changes, such as desensitization, that occur on a longer time scale than channel opening.

It follows from this model (Monod et al., 1965) that an activating allosteric effector of a channel-opening receptor is also, in principle, a channel-opening ligand (or agonist). This theoretical equivalence of effector sites and agonist sites results from the ion-flux rate (channel-opening equilibrium) being a direct measure of the conformational change, the energy of which is effected by both types of site. In this respect, channel-forming receptors differ from regulatory enzymes where catalysis occurs with specific substrates at the catalytic site and is not observed with only effectors binding at the regulatory sites. The ion-flux rate constant J is given by eq 9, where J_m is the maximal rate constant (which would pertain

$$J = J_m/[1 + T(1 + K_p/[P])^m(1 + K_l/[L])^n/(1 + \bar{K}_p/[P])^m(1 + \bar{K}_l/[L])^n] \quad (9)$$

if all the channels were open), [L] is the concentration of ligand

Table I: Parameters in Equation 9 Describing Enhancement of the Ion Flux Rate by Pentobarbital Concentrations below Inhibitory Concentrations (Values Used for the Calculated Lines in Figure 5)

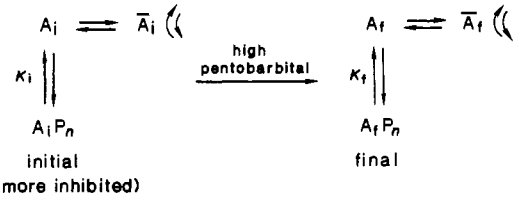
	faster desensitizing receptor (A)	slower desensitizing receptor (B)
max ion-flux rate constant (J_m) (all channels open) (s^{-1})	12.1[a]	2.55[a]
channel-closing equilibrium constant, $\Phi = [AL_2]/[\bar{A}L_2]$	0.27[a]	0.14[a]
ligand dissociation constants defined as microscopic (intrinsic) dissociation constants[b] dissociation constant of P from A or $\bar{A}$ is unaffected by the binding of L and the converse[d]		
K_l (μM)	142[a]	169[a]
$\bar{K}_l$ (μM)	<2	<2
K_p (μM)	250 ± 100	>5000
$\bar{K}_p$ (μM)	40 ± 15	80 ± 20
no. of pentobarbital binding sites (m)	2[c]	2
no. of GABA binding sites (n)	2[a]	2[a]

[a] Values from Cash and Subbarao (1987d). [b] As previously (Cash & Subbarao, 1987d), e.g., $K_p = 2[A][P]/[AP] = [A][P]/2[AP_2]$. [c] If the curve is fitted with $m = 1$, the values $\bar{K}_p = 20$ and $K_p > 1000$ are obtained. [d] Monod et al. (1965).

(GABA), and [P] is the concentration of effector (pentobarbital). Pentobarbital binds (heterotropically) at m sites, different from the GABA sites with dissociation constants K_p and $\bar{K}_p$ for the closed- and open-channel states, respectively. The ligand (GABA) binds to n sites with dissociation constants K_l and $\bar{K}_l$ for the closed- and open-channel forms, respectively. The constant T is related to the allosteric constant L' (Monod et al., 1965), $T = L'(\bar{K}_p/K_p)^m(\bar{K}_l/K_l)^n$. [The value of Φ (Cash & Subbarao, 1987d) is related by $\Phi = L'(\bar{K}_l/K_l)^2$.] Equation 9 illustrates the equivalence of the two ligands pentobarbital [P] and GABA [L]. The effectiveness of pentobarbital alone, on this receptor, in the absence of GABA depends on the values of K_p and $\bar{K}_p$.

The measured enhancement of chloride influx (Figure 5) at pentobarbital concentrations below where the inhibition became significant was fit by eq 9, with the values of the parameters given in Table I. In a previous study of the dependence of chloride-flux rates on GABA concentration, the number of GABA binding sites n for the best fit was shown to be 2 for both the receptors (Cash & Subbarao, 1987d). The values of J_B in Figure 5B required that the number of pentobarbital sites (m) mediating this enhancement of chloride flux is 2. The effect of the pentobarbital is apparently due to its binding to the open-channel form with a dissociation constant of 80 μM. This would cause an apparent increase in affinity for GABA and is consistent with an increased channel-open time. The effect of pentobarbital on the faster desensitizing receptor (J_A) was less steep than on J_B (Figure 5), and it was not possible to preclude a single barbiturate binding site in this case. Calculated on the basis of 2 pentobarbital sites ($m = 2$) pentobarbital was bound to the open-channel state of the faster desensitizing receptor with approximately the same affinity as it was to the slower desensitizing receptor. But in contrast to the slower desensitizing receptor, its dissociation constant from the closed form was only ca. 6 times higher than that from the open form (Table I). With the values of K_p and $\bar{K}_p$ it is possible to calculate values for the chloride-exchange rates J_B and J_A that would be obtained with pentobarbital in the absence of GABA. For both receptors eq 9 predicts a negligible ion-flux rate with pentobarbital alone, but a measurable chloride flux is predicted with pentobarbital in the presence of low GABA concentrations in the region of 1 μM GABA, which might enter the solution from endogenous sources (Figure 5, lowest curve). Pentobarbital is expected to shift the response/[GABA] curve to lower concentrations, as observed in these experiments. Correspondingly increasing concentrations of GABA shift the response/[pentobarbital] curve to lower pentobarbital concentrations (e.g., Figure 5).

The sharp inhibition of chloride exchange above ca. 200 μM pentobarbital in the second phase of ion flux, reaching complete inhibition by 1000 μM pentobarbital (Figures 5 and 6), is attributed to inhibition of channel opening of the slower desensitizing receptor. This inhibition is not decreased when the GABA concentration is increased to saturating values (Figures 5 and 6) and cannot be attributed to a decrease of GABA binding due to pentobarbital. This inhibition dominated the results of $^{36}Cl^-$-influx measurements in times up to a few seconds (Figure 1). But a recovery of the ion-flux activity was observed, in the presence of pentobarbital, and was characterized in three ways. (1) In longer times chloride-exchange activity recovered, giving rise to sigmoid-shaped ion-flux curves (Figures 1 and 3B). (2) After a short preincubation with pentobarbital, the initial rate of chloride exchange was greatly increased (Figure 3B). (3) The chloride influx in a constant time, a measure of the ion-flux activity (Cash & Subbarao, 1987c), was increased with increasing time of preincubation with pentobarbital (Figure 3A). It is not necessary to postulate a conformation change to explain inhibition of channel opening or desensitization by pentobarbital. The measurements of very rapid inhibition and the subsequent slower reactivation to a higher activity can be quantitatively explained by postulating the following model.



Pentobarbital binds to inhibitory sites of the receptor, with a dissociation constant higher than that associated with the activating sites, to give a receptor complex that will not form an open channel. This initial receptor–pentobarbital complex (A_iP_n), rearranges to a final conformation (A_fP_n), the activity of which is much less inhibited at that concentration of pentobarbital. (In this discussion, for simplicity, P_n refers to only the inhibitory bound pentobarbital molecules.) From numerous experiments of the three types, the activities of the initial (J_{Bi}) and final (J_{Bf}) mixtures of receptor in the presence of pentobarbital could be determined as well as the rates of relaxation of the initial to the final mixture. Above the pentobarbital concentration where inhibition occurs, the dependence of activity on pentobarbital concentration resolves into two curves (Figures 5B and 6C) corresponding to the A_i and A_f conformations. During exposure to pentobarbital the initial ion-flux activity J_{Bi}, corresponding to the A_i form, is converted

to J_{Bf} corresponding to the A_f form. The rate of this relaxation of the receptor–pentobarbital complex apparently has a very steep dependence on pentobarbital concentration increasing from 0.16 ± 0.12 s^{-1} with 1000 μM pentobarbital to 5.1 ± 1.2 s^{-1} at 2000 μM pentobarbital (Figure 4B). The pentobarbital-dependent equilibrium constant for inhibition can be estimated from the curves in Figures 5B and 6C and is unity at ca. 500 μM pentobarbital. Strictly speaking, these data do not rule out an activation by pentobarbital of previously inactive receptor while the fraction of total active receptor inhibited by pentobarbital remains the same. However, since the activation process is observed only at pentobarbital concentrations that inhibit the receptor, and since the reactivated activity never exceeds the original (with no inhibition by pentobarbital) activity, the conversion of the receptor to a less inhibited form is the simpler explanation. Either explanation involves pentobarbital-mediated conversion of one receptor form to another.

The dependence of the estimated values of J_{Bi} and J_{Bf} on pentobarbital concentration indicates that the smaller inhibition of the final form A_f is due to its ca. 3-fold decreased affinity for pentobarbital. Since a ligand-induced conformational change results in an increased affinity for that ligand, the recovery of activity and the inhibition must be mediated by different pentobarbital binding sites. This is consistent with the different dependence on pentobarbital concentration of the inhibition and reactivation rates. The steep dependence of reactivation rate on pentobarbital concentration (increasing 10-fold between 1000 and 2000 μM) indicates cooperativity involving a large number of pentobarbital molecules (>4). The pentobarbital concentration at which this reactivation process occurs is near that which causes a decrease of fluorescence intensity of tryptophan in membrane protein in synaptic plasma membrane (Harris & Schroeder, 1982). This effect has been attributed to a change in the membrane. Thus, it is conceivable that this change of the GABA receptor is related to a change in its interaction with the membrane.

The desensitization rate β was inhibited by pentobarbital, being approximately halved with 1000 μM pentobarbital (Figures 4A and 6B). The initial ion-flux rate constant for the final form J_{Bf} is inhibited in the same concentration range of pentobarbital. With these values governing the ion-flux rate throughout, there would be no loss of chloride influx in several seconds since the ratio J_{Bf}/β, on which depends the amplitude of $^{36}Cl^-$ influx (Cash & Subbarao, 1987d; Cash & Hess, 1984), is not reduced. However, the ion-flux rate J_{Bi} of the initial conformation is inhibited at ca. 3-fold lower pentobarbital concentrations (Figures 5B and 6B), and J_{Bi}/β is sharply reduced above 200 μM, where a marked inhibition of the second phase of chloride influx is seen in short times. It was not apparent that the desensitization rate β of the form A_i was inhibited more than that of A_f. Accordingly, eq 1, 4, and 5 were derived, assuming that both these forms were desensitized at the same rate, β.

The fast phase of ion flux mediated by the predominant, faster desensitizing receptor (Cash & Subbarao, 1987c,d) differed from the slow phase mediated by the slower desensitizing receptor in its inhibition by pentobarbital. The amplitude of $^{36}Cl^-$ influx was only slightly decreased at the highest pentobarbital concentrations. There is no evidence for complete inhibition and subsequent reactivation of J_A as described for J_B. However, these results cannot preclude the occurence of this type of behavior, with a much faster reactivation, completed in a shorter time than studied here (<40 ms).

Several measurements of GABA-mediated chloride isotope exchange in the presence of pentobarbital have been reported recently, and while this work was in progress. Variations in observation of inhibition of chloride ion exchange at high pentobarbital concentrations can be explained in the light of the reactivation following inhibition discovered here. Inhibition was not detected in $^{36}Cl^-$-efflux measurements having an assay time of ca. 10 s or more (Wong et al., 1984; Schwartz et al., 1984, 1985a). Some inhibition was observed in $^{36}Cl^-$-influx measurements with a 5-s assay time (Schwartz et al., 1986a,b), and somewhat greater inhibition was observed with an assay time of 3 s (Allan & Harris, 1986). Here we have determined the initial isotope-exchange activity, which is completely inhibited immediately after the addition of 1000 μM pentobarbital. We have also determined the activity after the reactivation (Figures 5 and 6), which occurs at a rate increasing steeply with pentobarbital concentration. When methods are used in which the determination of "activity" is an integral function of ion-exchange rate and specific internal volume (and desensitization rate), the apparent activity will increase with increased exposure to pentobarbital (during the assay or before). Figure 1B,C and 3B illustrate that the apparent inhibition of $^{36}Cl^-$ influx by high concentrations of pentobarbital, measured at a single $^{36}Cl^-$-influx time, depends on the assay time, decreasing with increasing time. Furthermore, the observation of complete inhibition would not be expected in measurements that include some chloride exchange due to the relatively uninhibited, faster desensitizing receptor. In the analyses presented here, the contributions of the two receptors are separated and activities defined as first-order rate constants are determined for the various species of receptor.

Inhibition of chloride conductance by similar, high concentrations of pentobarbital has been reported from several electrophysiological studies (Higashi & Nishi, 1981; Akaike et al., 1985; Parker et al., 1986; Connors, 1980; Simmonds, 1981). The similarity of pentobarbital concentrations for both the enhancing and the inhibitory effects on the chloride ion exchange measurements and the electrophysiological measurements (e.g., Akaike et al., 1985) supports the suggestion that these two methods are investigating the same phenomena.

At high pentobarbital concentrations, the binding of submicromolar concentrations of GABA to a membrane preparation was decreased with increasing pentobarbital concentration (Skerritt et al., 1983; Willow & Johnston, 1981b). This displacement of bound GABA was apparently from the so-called "low-affinity" sites (Skerritt et al., 1983). The pentobarbital concentration required for the inhibition of GABA binding depended on the techniques used in the preparation of the membrane suspension (Willow & Johnston, 1981b), and inhibition was not observed in all studies (e.g., Olsen & Snowman, 1982). The range of pentobarbital concentration (ca. 500–1000 μM) that caused a decrease in GABA binding was similar to that causing a significant reduction of the rates of desensitization of the slower desensitizing receptor, reported herein (Figures 4–6). The results reported here show that the inhibitions of chloride ion exchange and desensitization by pentobarbital were not due to decreased binding of GABA to the active state of the receptor. However, the similarity of the pentobarbital concentration displaying these two effects is consistent with a connection between them, and an explanation is suggested by the inhibition of desensitization. If the equilibrium of desensitization was shifted away from the desensitized state by pentobarbital binding to the sites that retard the desensitization rate, the high-affinity binding of GABA would be decreased, since the (relatively high-affinity) GABA

binding sites measured in these equilibrium binding measurements are those of the conformationally changed, desensitized receptor.

The different effects of pentobarbital on the fast and the slow phases of chloride exchange supports the conclusion that these are mediated by different receptors or different forms of the same receptor with different desensitization rates (Cash & Subbarao, 1987c,d). With anesthetic concentrations of pentobarbital both receptors displayed an increased channel-opening equilibrium and desensitization rate as a result of enhanced binding of GABA. For the predominant, faster desensitizing receptor the ratio of chloride ion exchange rate to desensitization rate was not increased, while for the slower desensitizing receptor it was increased significantly (Figures 4–6). With higher than anesthetic concentrations, channel opening of the faster desensitizing receptor was inhibited only slightly and desensitization was not inhibited in the concentration range studied. In contrast, channel opening of the slower desensitizing receptor was completely inhibited by 1 mM pentobarbital, although this was subsequently reactivated by a change in the receptor due to a high concentration of pentobarbital. The desensitization rate also of this receptor was decreased by high pentobarbital concentrations.

Pentobarbital can affect the activity of the GABA receptor in a number of different ways involving different binding sites. Besides an enhancement of channel opening and desensitization rate, higher concentrations of pentobarbital caused an inhibitory effect on these responses at least with one of the receptors. Finally, these experiments have revealed a conformational change other than channel opening or desensitization. This change affected the inhibitory effect that pentobarbital has on channel opening. The change to a less inhibited form was mediated by even higher concentrations of pentobarbital than the inhibition. Although these latter experiments were made with higher than normal pharmacological concentrations of the drug, they have enabled observations of changes that can occur in the receptor to modify its functional responses. The question arises whether natural substances in vivo can bind at these sites mediating this modulatory behavior.

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