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Channel Opening of γ-Aminobutyric Acid Receptor from Rat Brain: Molecular Mechanisms of the Receptor Responses†

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ABSTRACT: The function of γ-aminobutyric acid (GABA) receptors, which mediate transmembrane chloride flux, can be studied by use of $^{36}Cl^-$ isotope tracer with membrane from mammalian brain by quench-flow technique, with reaction times that allow resolution of the receptor desensitization rates from the ion flux rates. The rates of chloride exchange into the vesicles in the absence and presence of GABA were characterized with membrane from rat cerebral cortex. Unspecific $^{36}Cl^-$ influx was completed in three phases of ca. 3% ($t_{1/2}$ = 0.6 s), 56% ($t_{1/2}$ = 82 s), and 41% ($t_{1/2}$ = 23 min). GABA-mediated, specific chloride exchange occurred with 6.5% of the total vesicular internal volume. The GABA-dependent $^{36}Cl^-$ influx proceeded in two phases, each progressively slowed by desensitization. The measurements supported the presence of two distinguishable active GABA receptors on the same membrane mediating chloride exchange into the vesicles with initial first-order rate constants of 9.5 s^{-1} and 2.3 s^{-1} and desensitizing with first-order rate constants of 21 s^{-1} and 1.4 s^{-1}, respectively, at saturation. The half-response concentrations were similar for both receptors, 150 μM and 114 μM GABA for desensitization and 105 μM and 82 μM for chloride exchange, for the faster and slower desensitizing receptors, respectively. The two receptors were present in the activity ratio of ca. 4/1, similar to the ratio of "low-affinity" to "high-affinity" GABA sites found in ligand binding experiments. The desensitization rates have a different dependence on GABA concentration than the channel-opening equilibria. For both receptors, the measurements over a 2000-fold GABA concentration range required a minimal mechanism involving the occupation of both of the two GABA binding sites for significant channel opening; then the receptors were ca. 80% open. Similarly for both receptors, desensitization was mediated by a different pair of binding sites, although desensitization with only one ligand molecule bound could occur at a 20-fold slower rate.

The γ-aminobutyric acid (GABA) receptor is a membrane protein complex that, on binding GABA, forms transmembrane channels for chloride ion and thereby modifies the electrical potential of the membrane (Enna & Gallagher, 1983). The receptor has several binding sites for various drugs (Olsen, 1982; Johnson et al., 1984; Snodgrass, 1983) including the barbiturates and the benzodiazepines (Braestrup & Nielsen, 1983; Skolnick & Paul, 1982; Tallman & Gallager, 1985), which modulate its activity. A protein (Vaccarino et al., 1985) and a peptide (Ferrero et al., 1986) have been reported as endogenous modulators of its binding activity.

The functional responses of the receptor have been studied by electrophysiological techniques, and interactions between the binding sites have been studied by assaying the binding

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of radiolabeled substrates to membrane preparations (Enna & Gallagher, 1983; Olsen, 1982a,b). Ligand binding equilibria as measured in studies of response occur at higher concentrations than in direct measurements of binding. An understanding of the correlation of the results from the two methods is sought. Recently, measurements of transmembrane chloride flux with membrane vesicle preparations from brain have been reported (Schwartz et al., 1984; Sánchez et al., 1984; Subbarao & Cash, 1985; Allan et al., 1985; Harris & Allan, 1985). These methods allow the measurement of GABA receptor function in membrane preparations which can be mixed rapidly with solutions of known and controlled composition.

Channel opening and desensitization of acetylcholine receptors from electric fish have been studied with membrane preparations by rapid-mixing quench-flow techniques (Hess et al., 1983; Cash et al., 1985). We now report the use of quench-flow techniques to study functional responses of GABA receptor from mammalian brain. In preliminary accounts (Allan et al., 1985; Subbarao & Cash, 1985) we reported that the GABA-mediated chloride ion flux with a membrane preparation from rat brain proceeded in two rapid phases limited by two phases of desensitization. These two phases of desensitization (Cash & Subbarao, 1987a) could be resolved by the selective removal of the faster phase (Cash & Subbarao, 1987b) and were shown to correspond to two distinguishable receptors on the same membrane (Cash & Subbarao, 1987c). We now report rapid measurements of the chloride ion influx rate, which reflects the channel-opening equilibrium, over the complete GABA concentration range of response and compare the molecular mechanisms of the two receptors.

Materials and Methods

A membrane preparation from the cerebral cortex of male Sprague-Dawley rats, 4–6 weeks old, was prepared as described in the accompanying paper (Cash & Subbarao, 1987c). It was shown that the specific chloride influx measurements gave similar results when the Ficoll gradient separation was omitted, and some of the experiments were made with such a preparation. The suspension was adjusted to 750 μg of protein/mL, in solution B [145 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 10 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES), pH 7.5], for use in the experiments.

GABA-Mediated Chloride Ion Exchange. $^{36}Cl^-$ influx was performed in the quench-flow apparatus as described previously (Cash & Hess, 1981; Aoshima et al., 1981), at 30 °C, pH 7.5. The membrane vesicle preparation was kept at 0 °C. Each sample was warmed to 30 °C in 2 min after being loaded into the machine syringe and allowed to stand for a further 1 min before actuation. The membrane suspension (225 μL) was mixed with an equal volume of solution B containing GABA and $^{36}Cl^-$ (15 μCi/mL) (ICN Radiochemicals). After a predetermined incubation time, the specific ion uptake was terminated by being mixed with the same volume of bicuculline methiodide (Olsen et al., 1976) (3000 μM) in solution B. The mixture was passed immediately through a glass-fiber filter disc (Whatman GF/C). The vesicles retained on the filter disc were washed with solution B (3 × 10 mL), dried, and counted with toluene-based scintillation fluid. Unspecific uptake, measured in the same way in the absence of GABA, was subtracted from the total uptake to give the GABA-mediated, specific chloride influx. Typically, the measured specific signal at completion of ion flux was 2000 counts (10-min count) with a background increasing with time from 750 (≤100-ms incubation) to 2000 counts (3-s incubation). Each measurement was made in triplicate. Incubation times of ≤100 ms were achieved with continuous quench flow: times of ≥100 ms were achieved with pulse-mode quench flow (Cash & Hess, 1981; Fersht & Jakes, 1975). Equilibration of specific chloride uptake M_∞ was determined by allowing an incubation time of 6 s with 1000 μM GABA.

The quenching was shown to be sufficiently rapid in two ways. (a) No detectable specific chloride flux was observed when bicuculline methiodide was added with the GABA in times of 100 ms or 8 s, with preparations that gave a normal specific isotope uptake (typically 170 cpm) in the absence of bicuculline methiodide. Bicuculline methiodide was introduced with the GABA solution containing the $^{36}Cl^-$ to give the concentration normally present after addition of the quenching reagent. Solution B alone was added in the normal quenching syringe. In a control experiment the bicuculline was absent from the reaction mixture and was added at the normal time to give the same solution composition as the filter assay. Unspecific chloride flux was unaltered by bicuculline. (b) With a very short reaction time there was no observed specific chloride influx. The initiation and quenching time (after subtraction of dead time) was ≤3 ms. These experiments demonstrate that the specific chloride flux is reduced, by the added bicuculline or bicuculline methiodide, to a negligible rate in a time much shorter than that of the ion flux measurement.

Receptor Desensitization. The decrease in the GABA-mediated $^{36}Cl^-$ influx due to preincubation with GABA was followed as described in the accompanying paper (Cash & Subbarao, 1987c). Measurements were made at 30 °C, pH 7.5. The equilibration of specific $^{36}Cl^-$ influx, M_∞, was determined in each experiment by giving 6-s incubation with saturating GABA (1 or 10 mM). All determinations were made in triplicate. The unspecific background, measured in the same way in the absence of GABA, was subtracted from each point. The maximal specific influx was typically ca. 1500 counts on an unspecific background of ca. 1000 counts. The precision of the filter assay was typically ±75 counts or ±5% of the total specific volume.

Unspecific Chloride Ion Exchange. Uptake of $^{36}Cl^-$ was followed for times up to several hours with a protocol analogous to that used with the quench-flow machine. The membrane preparation (225 μL) in a test tube was brought to 30 °C by standing in a water bath for 3 min. Solution B containing $^{36}Cl^-$ (15 μCi/mL) (225 μL) was added to the membrane with vortex mixing. In addition, a measure of the specific chloride influx was made by including GABA (2 mM) in this solution. After the prescribed incubation time, solution B containing 3 mM bicuculline methiodide (225 μL) was added with vortex mixing. The mixture was passed through a glass-fiber filter disc (Whatman GF/C) and washed with solution B, and the radioactivity retained on the filter was measured as before.

Bicuculline Methiodide. Dried bicuculline (0.50 g) was added to a mixture of methylene chloride (10 mL) and methyl iodide (2 mL) in a 20-mL screw-top glass vial. The vial was tightly sealed, immediately shaken without heating to dissolve the solid, and allowed to stand for at least 24 h at room temperature. The precipitate was separated by suction filtration through sintered glass, washed with a little methylene chloride, and dried in vacuo with continuous evacuation for 2 h: yield 0.69 g (99%); mp 191–192 °C [lit. mp 180–181 °C (Olsen et al., 1976), 193 °C (softens), 203–207 °C (Pong & Graham, 1973)]; molar extinction coefficients $E_{\lambda=326nm}$ = 4331 M^{-1} cm^{-1}, $E_{\lambda=292nm}$ = 4347 M^{-1} cm^{-1}.

Results

The unspecific (GABA-independent) $^{36}Cl^-$ influx progressed

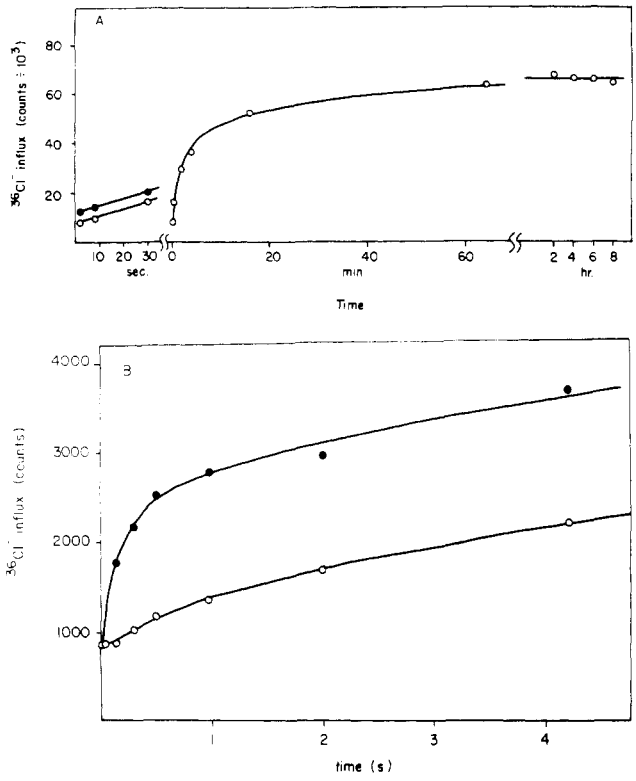

FIGURE 1: Unspecific $^{36}Cl^-$ influx into the membrane vesicles compared with GABA-specific influx at 30 °C, pH 7.5. (A) Measurements made in test tubes as described under Materials and Methods, following the unspecific $^{36}Cl^-$ influx (open symbols) to completion. Two major phases of unspecific chloride exchange are seen. The line is computed with eq 1 with $M_a(1 - e^{-k_a t}) = 0.1$, $M_b = 0.52$, $k_b = 0.0085\ s^{-1}$, $M_c = 0.38$, $k_c = 0.0005\ s^{-1}$, and $M_\infty = 66290$. When the measurement was made in the presence of GABA (1 mM) (filled symbols), the $^{36}Cl^-$ influx increased by an amount corresponding to 6.5% of the total internal volume. Each point is the mean of three determinations. The precision of the measurement was ±3% (standard deviation). (B) Measurements made in the quench-flow machine. The fastest phase of unspecific chloride exchange ($t_{1/2} = 0.6$ s) can be seen at the beginning of the second phase ($t_{1/2} = 82$ s) (open symbols). Omission of the membrane alone gives a filter background of 400 counts. The lowest unspecific flux measurement of 800 counts includes trapped radioactivity in the membrane preparation. In the presence of GABA (100 μM) (filled symbols), the measured counts increase due to chloride exchange with the GABA-specific population of membrane vesicles. The quench-flow measurements were made as described in the legend to Figure 3. Each point is the mean of three determinations. The precision of the measurements was ±75 counts (standard deviation).

in three phases, with half-times of 0.6 s, 82 s, and 23 min, respectively, reaching a final value which was stable for at least 8 h (Figure 1). The measurements were fitted to eq 1, where

$$\frac{M_t}{M_\infty} = M_a(1 - e^{-k_a t}) + M_b(1 - e^{-k_b t}) + M_c(1 - e^{-k_c t}) \qquad (1)$$

M_t/M_∞ is the fraction of the total chloride influx which has occurred at time t, M_a, M_b, and M_c are the fractions of the total exchange equilibrated in the three phases, and k_a, k_b, and k_c are first-order rate constants for the three phases, respectively. The first phase of unspecific influx (Figure 1B) varied in amplitude with the preparation and typically represented 2–3% of the total chloride exchange. The second and third phases (Figure 1A) represented two populations of approximately equal internal volume (56% and 41% of the total, respectively). A sample spun on the Ficoll gradient was somewhat richer in the less leaky population (36% and 61%, respectively). An initial fast phase of chloride exchange has been reported with membrane vesicles from *Torpedo californica* (White & Miller, 1981) and a synaptoneurosome preparation from rat brain (Schwartz et al., 1985). Extrapolation of the base line to zero time gave a value well above the filter blank (400 counts with a quench-flow experiment, e.g., Figure 1B), attributed to radioactivity occluded in the vesicles on the filter.

Figure 1 also shows the increase in $^{36}Cl^-$ uptake in the presence of GABA. This is completely inhibited by the GABA antagonist bicuculline or bicuculline methiodide and is attributed to specific GABA receptor mediated chloride exchange between the outside solution and 6.5% of the total internal volume. The total internal volume was 3.2 μL/mg of protein, and the GABA specific internal volume was 0.21 μL/mg. As shown in Figure 1B, the specific $^{36}Cl^-$ influx can be measured in short incubation times, after which the receptor channels are closed by mixing with bucuculline methiodide by quench-flow technique (Cash & Hess, 1981). In early experiments we used bicuculline, which was kept in unbuffered solution at pH 3 before being mixed in the quench-flow machine. It is more convenient to use bicuculline methiodide (Olsen et al., 1975, 1976; Pong & Graham, 1973, 1972; Johnston et al., 1972), which has the advantages of greater solubility, greater stability, and greater potency. A simple procedure is presented for the methylation of bicuculline in methylene chloride at room temperature giving a quantitative yield more conveniently than other methods.

In measurements of GABA-mediated events in this type of preparation, it should be shown that the results are not effected by the presence of endogenous GABA. The progress of the background (unspecific) chloride influx was not effected by the presence of the GABA antagonists, bicuculline (133 μM) or bicuculline methiodide (666 μM), indicating that this measurement is truly unspecific containing no contribution from chloride influx mediated by GABA. Second, it was shown that added GABA is not depleted by GABA uptake processes. The chloride influx was followed in the presence of guvacine (1 mM) (Schousboe et al., 1979) and nipecotic acid (1 mM) (Krogsgaard-Larsen & Johnston, 1975), each known to inhibit the sodium-dependent GABA uptake process at these concentrations. The flux curve was unaffected by these additives on the time scale of these experiments indicating that depletion of GABA concentration due to GABA uptake is not significant in these experiments.

The GABA-mediated uptake of $^{36}Cl^-$ by the membrane could be followed over its whole time course. The increase in counts was biphasic (Subbarao & Cash, 1985), with a fast phase of ion flux complete within 200 ms with saturating GABA and a remaining ion flux process which continued for a few seconds. With the lower GABA concentrations, complete equilibration of the chloride flux was not reached in these two phases of ion flux, indicating that both the phases of specific ion flux were terminated by receptor desensitization (Subbarao & Cash, 1985). This interpretation was tested by comparing the chloride flux curves with measurements of desensitization made independently with the same membrane preparation (Figure 2). Desensitization of GABA receptor incubated with GABA can be followed by the loss of $^{36}Cl^-$ uptake activity in a subsequent incubation (Cash & Subbarao, 1987c). Figure 2A shows chloride influx mediated by 40 μM GABA. The data are fitted to eq 2 in which M_t/M_∞ is the

$$\frac{M_t}{M_\infty} = 1 - \exp\left[-\left(J_A\frac{1 - e^{-\alpha t}}{\alpha} + J_B\frac{1 - e^{-\beta t}}{\beta}\right)\right] \qquad (2)$$

fraction of equilibration of ion flux into the vesicles, J_A and J_B are initial first-order rate constants for the two phases of

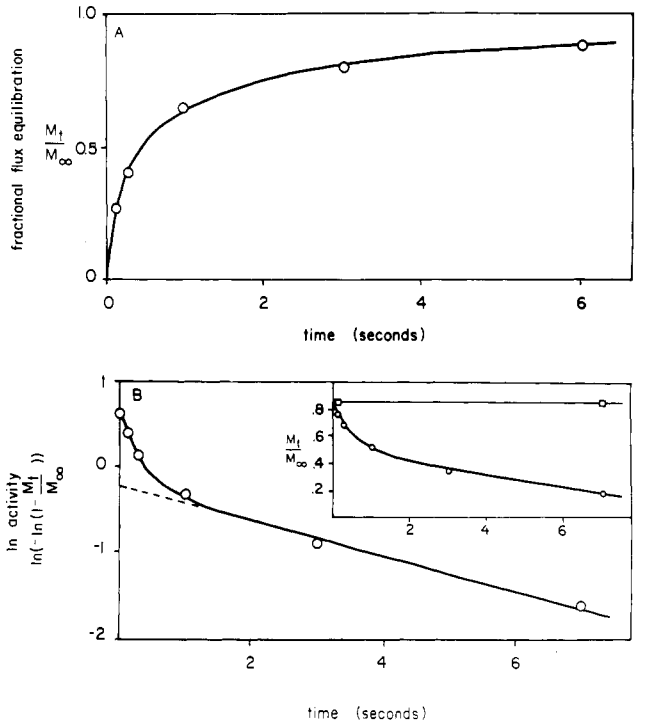

FIGURE 2: Comparison of the progress of chloride exchange with the progress of desensitization measured independently with the same preparation. (A) Influx of $^{36}Cl^-$ in the presence of 40 μM GABA. Measured as described in the legend to Figure 3. The points are means of three determinations. The line is a best fit computed from eq 2, giving the values $J_A = 2.22 \pm 0.5\ s^{-1}$, $\alpha = 4.00 \pm 0.7\ s^{-1}$, $J_B = 0.55 \pm 0.05\ s^{-1}$, and $\beta = 0.27 \pm 0.05\ s^{-1}$. The fast phase alone would give 0.43 of the equilibration. (B) First-order plot of the loss of activity (desensitization) in the presence of 40 μM GABA measured by following the loss of ion flux activity after progressive times of preincubation with GABA (Cash & Subbarao, 1987c). The negative slopes yield values of $\alpha = 3.8 \pm 0.4\ s^{-1}$ and $\beta = 0.21 \pm 0.03\ s^{-1}$. (Inset) The fractional equilibration (M_t/M_∞) of $^{36}Cl^-$ influx measured in a constant assay (1 mM GABA, 320 ms) after the various times of preincubation with GABA, indicated on the abscissa. The initial $^{36}Cl^-$ influx with no preincubation (squares) was obtained with no GABA present in the preincubation. The equilibration of $^{36}Cl^-$ influx (M_∞) was measured with no GABA in the preincubation and extending the assay time to 6 s.

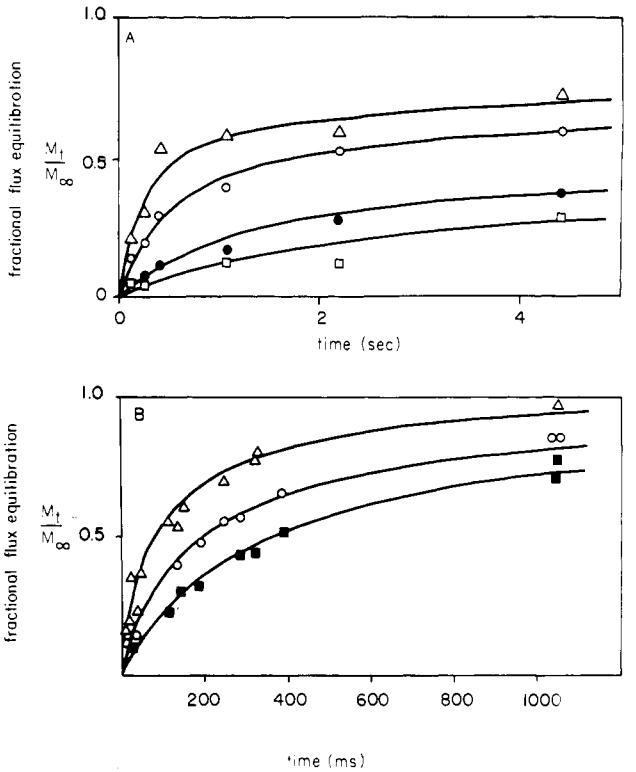

FIGURE 3: GABA-mediated $^{36}Cl^-$ flux into the membrane vesicles with a range of GABA concentrations. M_t/M_∞ is the fraction of complete chloride ion exchange that has occurred at the time on the abscissa. The influx was initiated by mixing the membrane suspension (225 μL) with an equal volume of solution B (Materials and Methods) containing $^{36}Cl^-$ (15 μCi/mL) and GABA. After the incubation time indicated on the abscissa, the specific exchange was terminated by mixing with the same volume of solution B containing bicuculline methiodide (3000 μM). The mixture was passed immediately through a glass-fiber filter disc, and the radioactivity retained on the filter was measured by scintillation counting. The unspecific isotope uptake was measured in the same way in the absence of GABA and subtracted to give the specific GABA-mediated influx. The points are the means of three determinations. The reproducibility was ±3% (standard deviation) of the total specific measurement. The lines are calculated from eq 2 with the values of the parameters indicated. (A) $^{36}Cl^-$ influx, with a single preparation, mediated by low concentrations of GABA: (□) 5 μM GABA, $J_A = 0.13\ s^{-1}$, $\alpha = 0.4\ s^{-1}$, $J_B = 0.013\ s^{-1}$, $\beta = 0.03\ s^{-1}$; (●) 10 μM GABA, $J_A = 0.27\ s^{-1}$, $\alpha = 0.7\ s^{-1}$, $J_B = 0.027\ s^{-1}$, $\beta = 0.07\ s^{-1}$; (○) 20 μM GABA, $J_A = 0.9\ s^{-1}$, $\alpha = 1.5\ s^{-1}$, $J_B = 0.09\ s^{-1}$, $\beta = 0.1\ s^{-1}$; (△) 40 μM GABA, $J_A = 2.1\ s^{-1}$, $\alpha = 3.0\ s^{-1}$, $J_B = 0.21\ s^{-1}$, $\beta = 0.3\ s^{-1}$. (B) $^{36}Cl^-$ influx mediated by high concentrations of GABA (different membrane preparations): (■) 40 μM GABA, $J_A = 2.0\ s^{-1}$, $\alpha = 3.0\ s^{-1}$, $J_B = 0.7\ s^{-1}$, $\beta = 0.18\ s^{-1}$; (○) 100 μM GABA, $J_A = 4.0\ s^{-1}$, $\alpha = 7.0\ s^{-1}$, $J_B = 1.4\ s^{-1}$, $\beta = 0.48\ s^{-1}$; (△) 1000 μM GABA, $J_A = 10.0\ s^{-1}$, $\alpha = 19.0\ s^{-1}$, $J_B = 3.5\ s^{-1}$, $\beta = 0.96\ s^{-1}$.

ion flux, and α and β are the first-order rate constants for desensitization of those phases, respectively. This curve fitting to the two phases of ion flux is possible because the faster ion flux process is the more rapidly desensitized and the two phases of desensitization are well separated in time. It is possible to determine values of α and β within limits of GABA concentration (a) when the chloride influx in the first phase is not too large and (b) when the amplitude of influx is not too low. Figure 2B shows the two phases of desensitization measured by the preincubation technique (Cash & Subbarao, 1987c). These two different methods gave values for α and β in good agreement.

The progress of the GABA-mediated transmembrane chloride flux was followed over a wide range of GABA concentration (2–10 000 μM). Examples are shown with low GABA concentrations in Figure 3A and high GABA concentrations in Figure 3B. The chloride influx measurements were fitted to the curves given by eq 2. The values of α and β obtained from the flux curves with intermediate concentrations of GABA (generally in the range 10–250 μM GABA) agreed with those measured by the preincubation method, independent of the ion flux time course (Cash & Subbarao, 1987c). Where α and β could not be obtained from the influx curves, the values of α and β measured with the preincubation method were used in the curve fitting to obtain values for J_A and J_B.

In summary, examination of this family of chloride influx curves revealed the following characteristics. (1) The rates of both $^{36}Cl^-$ influx and desensitization increased with increasing GABA concentration. (2) Influx occurred in two phases well separated in time, each becoming faster with increasing GABA concentration. (3) The values of the desensitization rates α and β, obtained by the curve fitting, where a phase is terminated by desensitization, agreed with those measured by the preincubation method (Cash & Subbarao, 1987c). (4) The amplitude (counts) of each phase increased with increasing GABA concentration. With relatively high GABA concentrations equilibration of chloride flux was achieved, but with low GABA concentrations the chloride exchange rate was reduced by desensitization to a rate negligible on this time scale before influx of $^{36}Cl^-$ was completed.

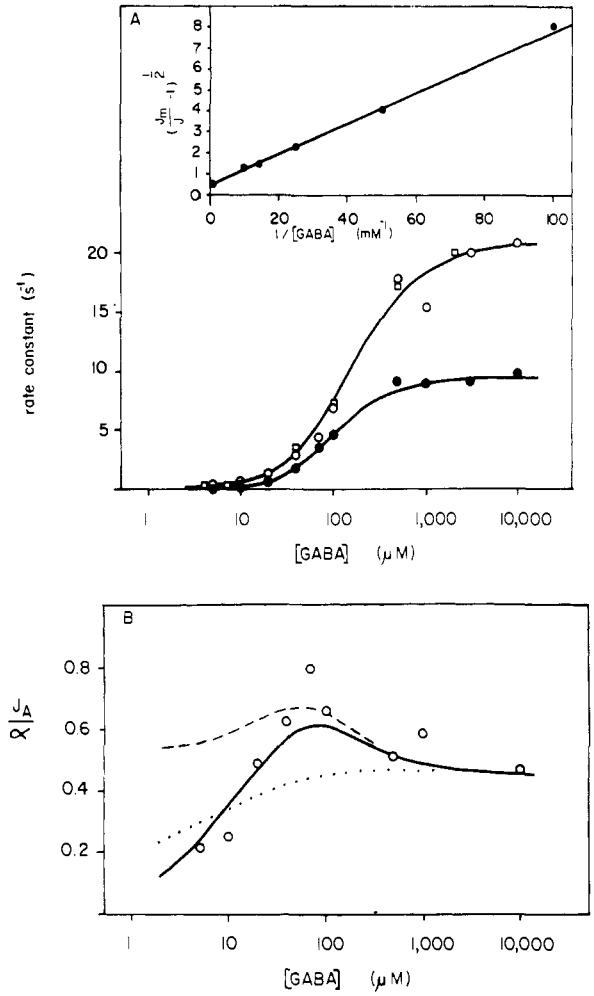

FIGURE 4: Dependence on GABA concentration of chloride exchange and desensitization for the faster desensitizing receptor. (A) Values of (●) J_A and (○) α from the fit of eq 2 to the influx curves compared with (□) α measured by the preincubation method (Cash & Subbarao, 1987c). (Inset) Linear plot of the influx data according to eq 6,

$$(J_m/J_A - 1)^{1/n} = \Phi^{1/n}K_1/L + \Phi^{1/n} \quad (6)$$

obtained by rearranging eq 4 (the parameters are defined in the text). J_m is adjusted to obtain the best fit to a straight line with a weighted least-squares linear regression program for chosen values of the integer n. The lines are calculated with the best fit parameters given in Table I. Φ and K_1 can be determined from the intercept and slope of this plot, optimally when the value of Φ is in the region of 1. (B) Variation of the ratio J_A/α with GABA concentration. The points were obtained by fitting eq 2 to the chloride influx curves. The continuous line is calculated from eq 4 and 5 and the parameters given in Table I. The dashed line corresponds to the same model without desensitization from the singly liganded species ($k_1 = 0$). The dotted line corresponds to the model with desensitization and channel opening mediated by the same binding sites (Cash & Hess, 1980).

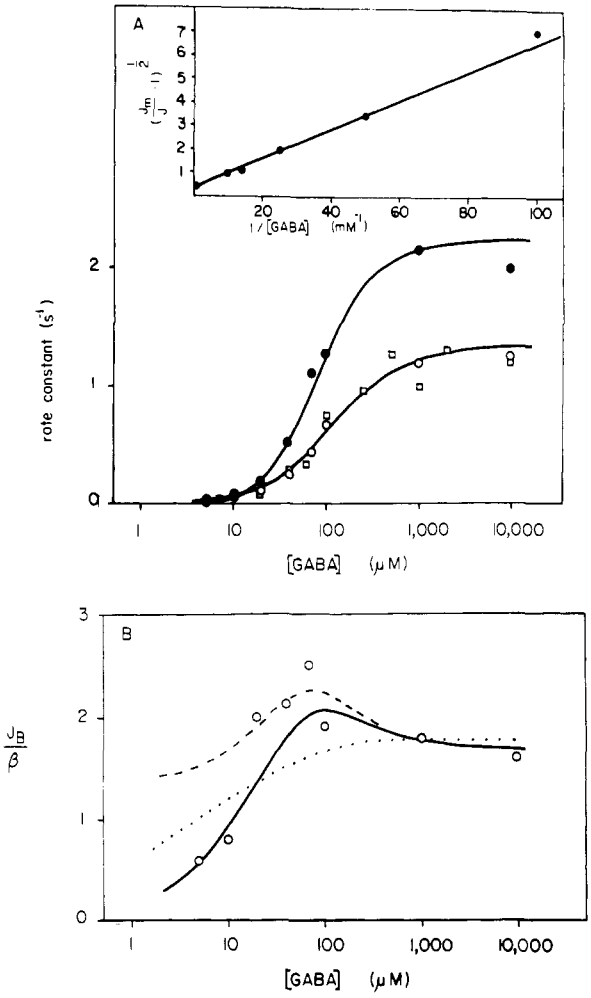

FIGURE 5: Dependence on GABA concentration of chloride exchange and desensitization for the slower desensitizing receptor. (A) Values of (●) J_B and (○) β from fitting eq 2 to the influx curves compared with (□) β measured by the preincubation method (Cash & Subbarao, 1987c). The lines are calculated from eq 4 and 5 and the parameters in Table I. (Inset) Linear plot of the influx data according to eq 6 as described in the legend to Figure 4. (B) Variation of the ratio J_B/β with GABA concentration. The points were obtained by fitting eq 2 to the influx curves. The continuous line is calculated from eq 4 and 5 and the parameters in Table I. The dashed and dotted lines represent less well fitting models as outlined in the legend to Figure 4.

(5) The ratio J_B/J_A was constant within experimental error, was independent of GABA concentration for a given rat brain preparation, and varied only within a small range for most preparations.

The rate constants J_A and J_B represent the initial ion flux activities for the two phases of ion flux. The finding that the ratio J_B/J_A was constant reflects a similar dependence on GABA concentration for the two phases. The differences in this ratio found in different preparations indicates that these activities can vary independently. Of 22 rat brain preparations, 20 gave similar values, $J_B/J_A = 0.27 \pm 0.12$, while 2 were extremely different with values of 0.02 and 1.64. Thus the initial fraction of activity in the slow phase was on average $J_B/(J_A + J_B) = 0.21$.

The values of J_A and J_B from experiments containing influx curves with several GABA concentrations were normalized to the mean value at 100 μM GABA, 4.59 ± 0.72 s^{-1} and 1.28 ± 0.30 s^{-1}, respectively, to correct for small activity differences between the preparations. The largest normalization was 30% in this series of experiments. The dependence of J_A on GABA concentration is plotted in Figure 4A together with the values of α. Similarly, Figure 5A shows the dependence on GABA concentration of J_B and β.

The increase of the amplitudes of chloride influx with increasing GABA concentration in each phase indicated that in both cases ion flux and desensitization had different dependencies on GABA concentration. The influx amplitude of each phase, after desensitization is complete, increases with the ratios J_A/α or J_B/β (eq 3) (Cash & Hess, 1984) but would remain constant if these ratios were unchanged. The differences in the dependencies on GABA concentration of ion flux and channel opening are small (Figures 4A and 5A) but are definitely required by the curve fitting analyses of the influx curves with different GABA concentrations. The variations of these ratios are shown in Figures 4B and 5B.

Discussion

The membrane preparation, prepared directly from the brain in the presence of protease inhibitors (Cash & Subbarao, 1987c), is expected to contain membrane closely related to that which transduces the neurotransmitter signals in vivo. This type of preparation contains sealed vesicular fragments of membrane. The suspension contained two major compartments that equilibrated with chloride ion in the solution with different rates ($k_b = 8.5 \times 10^{-3}$ s^{-1} and $k_c = 5 \times 10^{-4}$ s^{-1}), and it exhibited a very small rapid phase of equilibration with $k_a = 1.2$ s^{-1}.

Besides this unspecific chloride exchange (Figure 1), a small fraction (6.5%) of the internal volume gave chloride exchange, specifically in the presence of GABA, which was inhibited by GABA receptor antagonists. This specific chloride ion exchange occurred in two distinguishable phases. Each phase proceeded at a rate, and to an extent, that depended on the GABA concentration and is attributed to GABA receptor channels. It has previously been shown by analysis of desensitization kinetics that this preparation contains two distinguishable active GABA receptors situated on the same membrane (bounding a common population of vesicles) (Cash & Subbarao, 1987c). The desensitizations of these two receptors have similar dependences on GABA concentration but occur with a 16-fold difference in maximum rates. Analysis of the time course of the chloride ion exchange (Figure 2) confirmed that the two phases of chloride exchange are terminated by the two phases of desensitization with relatively low concentrations of GABA, hence are mediated by the faster and slower desensitizing receptors. First, the fractional equilibration of chloride influx was described by eq 1, and second, the rates of desensitization, α and β, determined from this equation agreed with those determined independently by a different type of experiment (Cash & Subbarao, 1987c).

The initial constants for ion flux, J_A and J_B, for the two receptors reflect the concentration of the receptors (with respect to internal volume), the fraction of them in the open channel state, and a rate constant for ion translocation through the open channel (Hess et al., 1981, 1980). The dependencies of these initial ion flux rate constants, derived from the chloride influx curves (e.g., Figure 3), on the concentration GABA shown in Figure 4 and 5 are similar to that of the desensitization rates. The similarity of the GABA concentration dependencies of J_A and J_B supports the evidence based on desensitization rates (Cash & Subbarao, 1987c) that ion flux is mediated by two distinguishable receptors. If the activity of a single ion channel is attenuated by a ligand concentration dependent process, the response of the remaining activity is expected to reach saturation at a significantly lower concentration than the original activity. This was not observed in these experiments.

At saturation with GABA the fractional activity of the slower desensitizing receptor, $J_B/(J_B + J_A)$ determined from the influx curves, was 0.19. This compares with the mean value of 0.16 estimated from the intercepts of the first-order plots of desensitization (Cash & Subbarao, 1987c). The mean value, over all concentrations in these chloride influx measurements, was 0.21. Comparing different rat brain preparations, there was some variation in the ratio J_B/J_A indicating that the values of J_A and J_B can vary independently. Occasionally a membrane preparation showed a large preponderance of slow or fast phase. However in general, approximately the same ratio of the two activities ($J_B/J_A = 0.27 \pm 0.12$) was found in the preparations. These measurements support the evidence (Cash & Subbarao, 1987c) that the more rapidly desensitizing receptor is the predominant one with ca. 4-fold greater concentration, if the channel-opening equilibria and the rates of ion translocation (Hess et al., 1981, 1980) do not greatly differ for the two receptors.

The rates of desensitization obtained from the flux curves agreed with those obtained independently in preincubation experiments (Cash & Subbarao, 1987c) (Figures 2, 4A, 5A). The ratios J_A/α and J_B/β were dependent on GABA concentration particularly below 40 μM GABA (Figures 4B and 5B). This reflects a different dependence on GABA concentration for the channel-opening equilibrium and the rate of desensitization. Although the effect appears small when the saturation curves of ion flux and desensitization are compared, the difference is consistent and well outside experimental error in the analysis of individual chloride flux curves. For example, the final fraction of the transmembrane chloride equilibration ($\bar{M}/M_\infty$) in each phase increases with GABA concentration at low concentrations (Figure 3), depending on the ratio J_A/α or J_B/β (eq 3, for the first phase) (Cash & Hess, 1984).

$$\frac{\bar{M}}{M_\infty} = 1 - \exp\left(-\frac{J_A}{\alpha}\right) \tag{3}$$

Considering the molecular mechanism of the channel-opening process, if there are n binding sites with equal affinity mediating channel opening (Figure 6a, $n = 2$) and channel opening is much larger with n activating ligand molecules bound than with $<n$, the ion-flux rate constant J is given by eq 4, where J_m is the flux rate constant that would obtain if

$$J = \frac{J_m}{1 + \Phi(1 + K_1/L)^n} \tag{4}$$

all the receptor channels were open, K_1 is the microscopic ligand dissociation constant, L is the ligand concentration, and Φ is the channel-closing equilibrium constant. The determined values of J_A and J_B were fitted to this model (Figures 4 and 5). In both cases the best fit was with $n = 2$. The fit was unacceptable for $n = 1$ and deteriorates as the integer n increases above 2. The constant Φ is a measure of the displacement of the second binding equilibrium by the channel-opening isomerization (Figure 6a) and is estimated from the change in the shape of the response curve due to that process (Figures 4A and 5A, inset) (Cash & Hess, 1980). The best fit value of Φ was similar for the two receptors (Table I) and corresponds to the doubly liganded receptor being mainly in the open-channel state (approximately 5/6 open). The minimal mechanism for channel opening and ion flux, that applies to both the receptors, is shown in Figure 6a.

Two GABA binding sites mediating desensitization are required to explain its dependence on GABA concentration as was found previously for each receptor (Cash & Subbarao, 1987c). Measurements of cation flux mediated by the acetylcholine receptor were found to be consistent with a minimal kinetic scheme in which channel opening and desensitization were mediated by binding of neurotransmitter at the same two sites (Cash & Hess, 1980; Aoshima et al., 1980; Cash et al., 1981). The different dependence on acetylcholine concentration of these two responses could be explained by the occurrence of desensitization with only one binding site occupied. It is of interest whether a similar mechanism could apply to the GABA receptors under study here. Attempts to fit this common binding site mechanism to our results for both receptors led to the prediction of a slightly (ca. 1.3-fold) lower half-response concentration for desensitization than was observed. The results could be better described by a scheme involving different binding sites mediating desensitization and

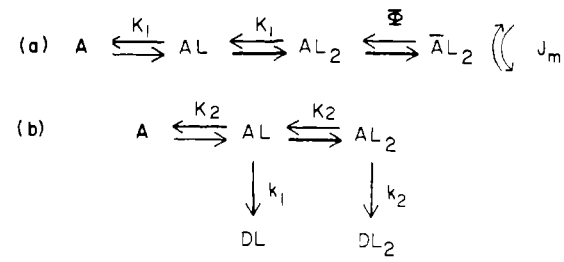

FIGURE 6: Minimal mechanisms to account for the dependence on GABA concentration of (a) channel opening and ion flux and (b) receptor desensitization. The same minimal mechanisms apply to both receptors. For convenience the same symbols for the microscopic reaction steps are used for each receptor (Table I, eq 4–6). (a) Ion flux. An active state of the receptor, A, can bind two ligand molecules with microscopic dissociation constants, K_1, to give a doubly liganded species, AL_2, which isomerizes to give an open channel state, $\overline{AL_2}$, with a channel-opening equilibrium constant Φ^{-1}. Ion flux through the open channel proceeds with a rate constant J_m. (b) Desensitization. An active state of the receptor, A, can bind two ligand molecules with microscopic dissociation constants, K_2. The singly liganded species, AL, and the double liganded species, AL_2, are converted (desensitized) with rate constants k_1 and k_2, respectively, to inactive states, D, which do not form open channels. The reversal of desensitization or the binding equilibria of the desensitized species and the formation of additional states of the receptor are not indicated merely because they do not contribute to the results reported here.

channel opening. A stronger argument for different sites comes from a consideration of the ratios J_A/α and J_B/β determined within individual experiments (Figures 4B and 5B). As would be predicted by the mechanisms a and b in Figure 6, the response curves for ion flux and desensitization are different shapes (even if $k_1 = 0$), giving rise to varying ratios of J_A/α and J_B/β. However to explain the magnitude of these variations, the occurrence of relatively slow desensitization with only one of the two GABA binding sites occupied is required. The ratios J_A/α and J_B/β derived from fitting the results of individual experiments reflect the different ligand concentration dependencies of the channel-opening equilibrium and desensitization rates and are a sensitive test of the molecular mechanism. The response curves for desensitization of each of the two receptors are described by eq 5, derived from the

$$\alpha \text{ or } \beta = \frac{k_2 + 2K_2k_1/L}{(1 + K_2/L)^2} \qquad (5)$$

minimal mechanism shown in Figure 6b, where K_2 is the microscopic ligand dissociation constant, L is the ligand concentration, and k_1 and k_2 are the specific reaction rates for desensitization of the singly liganded and doubly liganded receptor, respectively.

The values of the constants obtained by fitting the measurements to the minimal mechanisms in Figure 6 are given for each receptor in Table I. The concentration range of response is similar for both receptors and for desensitization as well as channel opening. The higher value for the microscopic constant K_1, compared with K_2, reflects the cooperative effect on ligand binding due to the channel-opening isomerization. Since the channel-opening equilibria of the two receptors are comparable, the higher chloride exchange rate mediated by the faster desensitizing receptor must be due to a higher ion translocation rate or a larger concentration of receptor. If the chloride translocation rates are comparable, the faster desensitizing receptor is 4–5-fold higher in concentration than the slower desensitizing one.

The GABA concentration range of the major part of the channel-opening response of the two receptors was between 10 and 1000 μM. Electrophysiological results have indicated a slightly higher concentration range with ganglia (Gallagher et al., 1983, 1978), a similar concentration range with rat brain receptor expressed in oocytes (Houamed et al., 1984) and with crustacean muscle (Feltz, 1971; Brookes & Werman, 1973), and a slightly lower concentration range with rat (Parker et al., 1986) and chick (Miledi et al., 1982; Smart et al., 1983) brain receptor expressed in oocytes, with hippocampal neurons (Ozawa & Yuzaki, 1984), spinal neurons (Choi & Fischbach, 1981), and ganglia (Akaike et al., 1985), and with crayfish muscle (Takeuchi, 1969; Dudel & Hatt, 1976). Chloride ion flux experiments have indicated a slightly higher concentration range with hippocampal slices (Wong et al., 1984), a similar concentration range with crayfish muscle (Ticku & Olsen, 1977), and a slightly lower concentration range with a membrane preparation from mouse brain (Allan & Harris, 1986; Harris & Allan, 1985). One major experimental problem has been the occurrence of desensitization, which increases with increasing GABA concentration, shifting the concentration dependence to lower concentrations and decreasing the maximum response. An additional problem in experiments where diffusion is important is estimating the ligand concentration at the time the response is measured. In ion flux measurements, if extremely short incubation times are not given, the extent of ion exchange depends on the desensitization rate as well as the channel-opening process. Furthermore if conditions are reached, in which the ion exchange into the vesicles approaches equilibration in the incubation time, no further increase in counts may be seen, even when the open-channel concentration continues to increase with increasing neurotransmitter. In these analyses of quench-flow chloride ion influx curves, the ion flux rate constants, which are a measure of the channel-opening equlibrium, have initial values independent of desensitization. In quench-flow experiments the solutions are mixed in a time much shorter than the times at which the measurements are made.

In the mechanism which best fit the results, the receptor has two ligand binding sites which must both be occupied for significant channel opening (Figure 6a). The binding of two GABA molecules for channel opening was suggested by measurements with ganglion cells (Akaike et al., 1985) and crayfish muscle (Takeuchi & Takeuchi, 1969) and at least two with spinal neurons (Sakmann et al., 1983), but other reports have favored three (Gallagher et al., 1978; Brookes & Werman, 1973) or four (Feltz, 1971) GABA molecules. Values of the Hill coefficient near 1.8 have been reported [e.g., Akaike et al. (1985), Ticku and Olsen (1977), and Choi and Fischbach (1981)].

The sodium-independent binding of GABA to preparations from brain homogenate which is attributed to GABA receptor (Peck et al., 1973; Enna & Snyder, 1975; Zukin et al., 1974) has been the basis of several studies of the GABA receptor (Enna & Gallagher, 1983). Binding of GABA to a high-affinity site with a dissociation constant (K_d) in the region of 10–20 nM, a low-affinity site with a K_d in the region of 100–200 nM, and a lower affinity site with a K_d in the region of 1 μM has been reported (Olsen, 1982b; Falch & Krogsgaard-Larsen, 1982). These affinities are much greater than those of the binding processes which mediate the functional responses of the GABA receptor. However, this does not mean that the sites measured in the GABA-binding assays are unrelated to the functional response. If a conformational change, for example, desensitization, is mediated by ligand binding, the affinity of the binding site must be increased by this change (Katz & Thesleff, 1957; Monod et al., 1965; Koshland et al., 1966; Hammes & Wu, 1974). This is true for any model of allosteric transitions. Thus, the ligand concentration depen-

Table I: Comparison of the Two Receptors: Parameters That Summarize the Measurements with GABA and That Pertain to Figure 6 and Equations 4 and 5

	parameter	faster desensitizing receptor (A)	slower desensitizing receptor (B)
(a) chloride ion flux	J_{max} (s^{-1})	9.5 ± 0.7	2.25 ± 0.15
	$J_m = J_{max}(1 + \Phi)$ (s^{-1})	12.1 ± 2.1	2.55 ± 0.45
	half-response GABA concentration (μM)	105 ± 10	82 ± 9
	$K_1 = 2[A][L]/[AL] = [AL][L]/2[AL_2]$ (μM)	142 ± 50	169 ± 60
	$\Phi = [AL_2]/[\overline{AL_2}]$ (closed/open)	0.27 ± 0.15	0.14 ± 0.11
	$\Phi^{1/2}K_1$ (μM)	73 ± 7	62 ± 5
(b) desensitization	k_2 (s^{-1})	α_{max} = 21 ± 2.2	β_{max} = 1.35 ± 0.18
	k_1 (s^{-1})	1.0 ± 0.2	0.1 ± 0.026
	half-response GABA concentration (μM)	151 ± 25	114 ± 14
	$K_2 = 2[A][L]/[AL] = [AL][L]/2[AL_2]$ (μM)	70 ± 9	53 ± 7

dence of the *rate* of desensitization follows that of ligand binding to the initial protein conformation, while the ligand concentration dependence of equilibrium binding is at lower concentrations, following that of ligand binding to the desensitized conformation, if the conformational conversion is complete. Binding sites of low affinity, such as those mediating the functional responses, have not been detected in binding assays for experimental reasons. (a) At higher radioligand concentrations the background is high and the signal/background ratio is too low. (b) Dissociation is rapid and can be significant during removal of the radioligand, for example, when the receptor preparation is washed on a filter disc. (c) Ligand-induced conformational transitions which change the binding site (e.g., desensitization) are rapid and can be significant in the time of the binding assay. But low-affinity sites would be deteced as higher affinity sites in so far as the duration of the binding assay exceeds that of the ligand-induced conformational change.

The ratio of the concentration of the low-affinity to the high-affinity GABA sites in mammalian brain determined in the binding assay is similar to the ratio of the faster to the slower desensitizing receptors described here. For example, ratios of 5–8 for rat cortex (Guidotti et al., 1979; Falch & Krogsgaard-Larsen, 1982) and 6 for bovine cortex (Olsen & Snowman, 1982) have been reported. Moreover, the two types of sites identified in the binding assays probably represent two independent classes of GABA receptor (Guidotti et al., 1979), as suggested by their varying stoichiometry with brain region and also after denervation, by the differential modulation of the ligand binding by GABA-modulin, and by differential susceptibility of ligand binding to chemical reagents (Maksay & Ticku, 1984a,b). Identification of these relatively high-affinity binding sites with the functional sites of the two receptors observed here should be made cautiously. Such correlations are difficult for several reasons. (a) The sample preparation methods are different for the binding measurements than for measurements of ion flux. (b) Binding measurements include any kind of GABA receptor including those with channels for ions other than chloride. (c) Desensitization would decrease the GABA dissociation constant to an extent increasing with the equilibrium of desensitization, and the order of binding affinities need not be the same after as before desensitization.

It is normally difficult to distinguish binding sites which mediate different responses but have similar dependencies on ligand concentration. However, the measured variations of the ratios of channel-opening equilibrium and desensitization rate (Figures 4B and 5B) favor separate binding sites for these two processes. Since both desensitization and channel opening are mediated by two binding sites, the responses described here would be mediated by four GABA binding sites on each receptor. The channel-opening sites are of too low affinity to be detected in the reported measurements of GABA binding, unless further ligand-induced conformational changes, not detected in this work, occur. The desensitization sites might have a sufficiently increased affinity after desensitization to be detected in the binding measurements, as discussed above.

The GABA receptors studied here have some similarities with the acetylcholine receptor studied by ion flux methods (Cash & Hess, 1980; Aoshima et al., 1980; Cash et al., 1981). The response concentrations are similar and relatively high (half-response concentration $\simeq$ 100 μM), suitable for transducing a synaptic signal, where release of a high concentration of neurotransmitter gives a high response rate with a high local concentration of receptors, and a high signal termination rate is allowed by a high dissociation rate when the concentration of neurotransmitter in solution falls. The receptors are desensitized rapidly. Desensitization rate follows a different dependence on neurotransmitter concentration than channel-opening equilibrium, becoming relatively more important at low concentrations. Desensitization can occur without channel opening and with only one bound neurotransmitter molecule. It remains to be seen whether these are general properties of neurotransmitter receptors in a signal transducing membrane.

Apparently, for the GABA receptor, channel opening is not an obligatory intermediate for desensitization. Desensitization can proceed at GABA concentrations at which the channels are closed. It is possible that in some circumstances the limiting factor in the removal of GABA is GABA uptake when depletion by diffusion is slow (Dingledine & Korn, 1985; Mathews et al., 1981). In such a case, with a persistent, low GABA concentration, desensitization could continue in the absence of channel opening.

The GABA receptors studied in this work might be related in various ways (Cash & Subbarao, 1987c). They could be different forms of the same protein although different proteins with similar behavior are not at present ruled out. It is a possibility that these two receptors, with a 16-fold difference in mean active lifetimes, function together on the signal transducing membrane with complementary roles in neurotransmission.

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