Rogart, R. B. (1986) *Ann. N.Y. Acad. Sci. 479*, 402–430.
Rogers, R. S., & Rapoport, H. (1980) *J. Am. Chem. Soc. 102*, 7335–7339.
Rosemblatt, M., Hidalgo, C., Vergara, C., & Ikemoto, N. (1981) *J. Biol. Chem. 256*, 8140–8148.
Shimizu, Y., Hsu, C., Fallon, W. E., Oshima, Y., Miura, I., & Nokanishi, K. (1978) *J. Am. Chem. Soc. 100*, 6791–6793.
Shimizu, Y., Hsu, C., & Genenah, A. (1981) *J. Am. Chem. Soc. 103*, 605–609.
Shull, G. E., Greeb, J., & Lingrel, J. B. (1986) *Biochemistry 25*, 8125–8132.
Sigworth, F. J. (1983) in *Single Channel Recording* (Sakmann, B., & Neher, E., Eds.) pp 3–35, Plenum, New York.
Sigworth, F. J., & Spalding, B. C. (1980) *Nature (London) 283*, 293–295.
Spalding, B. C. (1980) *J. Physiol. (London) 305*, 485–500.
Strichartz, G. (1984) *J. Gen. Physiol. 84*, 281–305.
Strichartz, G., Rando, T., Hall, S., Gitschier, J., Hall, L., Magnani, B., & Hansen-Bay, C. (1986) *Ann. N.Y. Acad. Sci. 479*, 96–112.
Van Alstyne, E., Burch, R. M., Knickelbein, R. G., Hungerford, R. T., Gower, E. J., Webb, J. G., Poe, S. L., & Lindenmayer, G. E. (1980) *Biochim. Biophys. Acta 602*, 131–143.
Weiss, R. E., & Horn, R. (1986) *Science (Washington, D.C.) 233*, 361–364.
Wollner, D. A., & Catterall, W. A. (1985) *Brain Res. 331*, 145–149.
Woodhull, A. M. (1973) *J. Gen. Physiol. 61*, 687–708.
Worley, J. F., III, French, R. J., & Krueger, B. K. (1986) *J. Gen. Physiol. 87*, 327–349.

Desensitization of γ-Aminobutyric Acid Receptor from Rat Brain: Two Distinguishable Receptors on the Same Membrane†

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Received May 12, 1987; Revised Manuscript Received August 11, 1987

ABSTRACT: Transmembrane chloride flux mediated by γ-aminobutyric acid (GABA) receptor can be measured with a mammalian brain homogenate preparation containing sealed membrane vesicles. The preparation can be mixed rapidly with solutions of defined composition. Influx of $^{36}Cl^-$ tracer initiated by mixing with GABA was rapidly terminated by mixing with bicuculline methiodide. The decrease in the isotope influx measurement due to prior incubation of the vesicle preparation with GABA, which increased with preincubation time and GABA concentration, was attributed to desensitization of the GABA receptor. By varying the time of preincubation with GABA between 10 ms and 50 s with quench-flow technique, the desensitization rates could be measured over their whole time course independently of the chloride ion flux rate. Most of the receptor activity decreased in a fast phase of desensitization complete in 200 ms ($t_{1/2}$ = 32 ms) at saturation with GABA. Remaining activity was desensitized in a few seconds ($t_{1/2}$ = 533 ms). These two phases of desensitization were each kinetically first order and were shown to correspond with two distinguishable GABA receptors on the same membrane. The receptor activities could be estimated, and the faster desensitizing receptor was the predominant one, giving on average ca. 80% of the total activity. The half-response concentrations were similar, 150 and 114 μM for the major and minor receptors, respectively. The dependence on GABA concentration indicated that desensitization is mediated by two GABA binding sites. The fast desensitization rate was approximately 20-fold faster than previously reported rates while the slower desensitization rate was slightly faster than previously reported rates.

The γ-aminobutyric acid (GABA) receptor is the major inhibitory neurotransmitter receptor in the mammalian brain. It is crucial in controlling the excitability of neurones (Enna & Gallagher, 1983; Krnjevic, 1974). Electrophysiological experiments showed that a GABA-induced increase in transmembrane potential at postsynaptic membrane is caused by an increase in its permeability to chloride ion. Measurements with radioactive chloride have supported this, with brain slices (Wong et al., 1984) and cultured neurones (Thampy & Barnes, 1984). Subsequently, GABA-mediated transmembrane chloride flux was demonstrated with membrane preparations containing vesicles from brain homogenate of rat or mouse (Allan et al., 1985; Subbarao & Cash, 1985; Sânchez et al., 1984; Schwartz et al., 1986a). The pharmacology of this response corresponded with the $GABA_A$-type receptor (Harris & Allan, 1985). This type of preparation can be mixed rapidly with solutions of known composition, allowing biochemical studies of the responses of the GABA receptor to be made.

A number of channel-forming neurotransmitter receptors are desensitized by their neurotransmitter. During exposure to the neurotransmitter, in times much longer than the channel-opening reaction, the receptor is transformed to a state that does not form open channels. Desensitization of GABA receptor has been observed with electrophysiological techniques (Krnjevic, 1981) in hippocampal neurones (Ozawa & Yuzaki, 1984; Numann & Wong, 1984; Thalman & Hershkowitz, 1985), in ganglion cells (Gallagher et al., 1983; Adams & Brown, 1975; Hackman et al., 1982; Akaiki et al., 1985), in rat brain receptor expressed in oocytes (Houamed et al., 1984;

†This work was supported in part by a grant from the Research Council of the University of Missouri—Columbia Medical School.
‡Recipient of a Missouri Institute of Psychiatry fellowship.

0006-2960/87/0426-7556$01.50/0

Parker et al., 1986), and in crustacean muscle (Dudel & Hatt, 1976; Epstein & Grundfest, 1970; Sarne, 1976). In other experiments no desensitization of GABA receptor was observed (Dudel & Hatt, 1976; Ticku & Olsen, 1977; Thampy & Barnes, 1984). The desensitization of acetylcholine receptor from the electric organs of fish was studied with membrane vesicle preparations by a rapid-mixing technique (Hess et al., 1979; Cash & Hess, 1981) by measuring the decrease in acetylcholine-mediated cation flux after incubation of the receptor with acetylcholine for varying times (Hess et al., 1979; Aoshima et al., 1980, 1981). These studies established the ligand concentration dependence of this desensitization.

GABA-mediated transmembrane chloride flux, in times between 5 ms and several seconds, has been measured with a membrane preparation from rat brain by quench-flow techniques. Rapid desensitization of the GABA receptor was reported (Allan et al., 1985; Subbarao & Cash, 1985). Desensitization occurred in two distinct phases within the time regions of 100 ms and 2 s, respectively (Subbarao & Cash, 1985; Cash & Subbarao, 1987b). We now report the application of rapid-mixing, sequential incubation experiments to the study of desensitizations of GABA receptor in native membrane freshly prepared from rat brain, over a wide range of GABA concentration. In this paper we show that the measurements characterize the rapid desensitization of two distinguishable receptors on the same membrane. These conclusions are the basis of the interpretation of measurements of the time course of the GABA-mediated chloride ion exchange, described in the accompanying paper (Cash & Subbarao, 1987a), which reflect the channel-opening equilibrium.

Experimental Procedures

Preparation of Membrane Vesicle Suspension. Male Sprague-Dawley rats, 4–6 weeks old, were killed by decapitation with a guillotine. All manipulations were performed on ice, and solutions were at 0–4 °C. The cerebral cortex was cleaned by rinsing with cold saline, cut into 1-mm slices, and suspended in 30 mL of 0.32 M sucrose–10 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES) (Calbiochem), pH 7.5 (solution A), containing the protease inhibitors phenylmethanesulfonyl fluoride (1 mM), aprotinin (10 μg/mL), antipain (5 μg/mL), leupeptin (5 μg/mL), and pepstatin (5 μg/mL) (Sigma Chemical Co.) and the antioxidant butylated hydroxytoluene (20 μM). The mixture was homogenized with a Virtis 45 homogenizer (setting 30) for 5 s. An equal volume of solution B (145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, 10 mM HEPES, pH 7.5) was added with gentle stirring, and the mixture was centrifuged for 4 min, 270*g*. The supernatant was centrifuged for 30 min, 23640*g*. The pellet was resuspended in 8 mL of solution B, with a glass–Teflon hand homogenizer, layered on a 4–12% Ficoll gradient in solution A, and centrifuged for 1 h, 110000*g*. The middle band (9–10% Ficoll) was diluted with 2 volumes of solution B and centrifuged for 30 min, 35000*g*. The pellets were resuspended in solution B and adjusted to 750 μg of protein/mL. The protein concentration was assayed by the bicinchoninic acid method (Smith et al., 1985) (Pierce Chemical Co.). Similar results were obtained when the Ficoll gradient separation was omitted.

Desensitization of the Receptor. By use of the quench-flow technique the membrane vesicle suspension (225 μL) was mixed with an equal volume of solution B containing GABA. After a predetermined preincubation time the suspension was mixed with solution B (225 μL) containing GABA (30 mM) and $^{36}Cl^-$ (20 μCi/mL), and the $^{36}Cl^-$ influx in 320 ms was measured in a second incubation as described in the accompanying paper (Cash & Subbarao, 1987a). Typically, the initial specific signal was ca. 2000 counts/10 m, and the background of ca. 1000 counts/10 m was constant in the experiment. The zero preincubation time points were obtained with no GABA in the preincubation solution, and this value was independent of preincubation time t_p, indicating that the vesicles remained intact during the measurement. The equilibrium value of the chloride influx M_∞ was determined by omitting GABA from the preincubation and extending the second incubation time to 6 s. The unspecific flux base line was determined by omitting GABA from both incubations.

In control experiments to check the stability of the preparation during the whole experimental session, the M_∞ and base-line points were measured at the end, as well as the beginning, of each experiment. Normally there was no decrease in the signal. Occasionally there was a decrease in internal volume of up to 10% in the whole session (ca. 6 h). If such experiments were used, a small correction for this was applied.

Treatment of Data. The loss of receptor activity during incubation with neurotransmitter can be followed in a subsequent ion flux assay (Aoshima et al., 1981). If two types of GABA receptor on the same population of membrane vesicles mediate chloride exchange through ion channels, the specific $^{36}Cl^-$ influx into the vesicles is described by eq 1, where

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

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

$$J_B = J_{B(t_p=0)}e^{-\beta_p t_p} \quad (3)$$

M_t/M_∞ is the fractional equilibration of $^{36}Cl^-$, J_A and J_B are initial first-order rate constants for chloride exchange, α and β are first-order rate constants for desensitization of the two receptors, respectively, and t is the incubation time. After some desensitization of the receptors in a preincubation with GABA, the values of J_A and J_B are given by eq 2 and 3, where the subscript p denotes the preincubation. Equations 1–3 give rise to eq 4, which describes the decrease in $^{36}Cl^-$ influx due to a

$$-\ln\left(1-\frac{M_t}{M_\infty}\right) = Ae^{-\alpha_p t_p} + Be^{-\beta_p t_p} \quad (4)$$

$$A = J_{A(t_p=0)}\frac{1-e^{-\alpha t}}{\alpha} \quad (5)$$

$$B = J_{B(t_p=0)}\frac{1-e^{-\beta t}}{\beta} \quad (6)$$

preincubation with GABA. In the present case, since $\alpha \gg \beta$, the desensitization occurs in two distinct phases with amplitudes represented by A and B (eq 5 and 6), which are constants since the assay (second) incubation has constant conditions and duration.

A first-order plot, according to eq 4, of ln ion flux activity, ln [− ln (1 − M_t/M_∞)], against t_p gives a line, the linear portions of which have negative slopes giving the rate constants $\alpha + \beta$ and β. The ordinate intercept of the faster phase $I_{(A+B)}$ is given by eq 7 and that of the slower phase I_B by eq 8. These

$$I_{(A+B)} = \ln\left(J_{A(t_p=0)}\frac{1-e^{-\alpha t}}{\alpha} + J_{B(t_p=0)}\frac{1-e^{-\beta t}}{\beta}\right) \quad (7)$$

$$I_B = \ln\left(J_{B(t_p=0)}\frac{1-e^{-\beta t}}{\beta}\right) \quad (8)$$

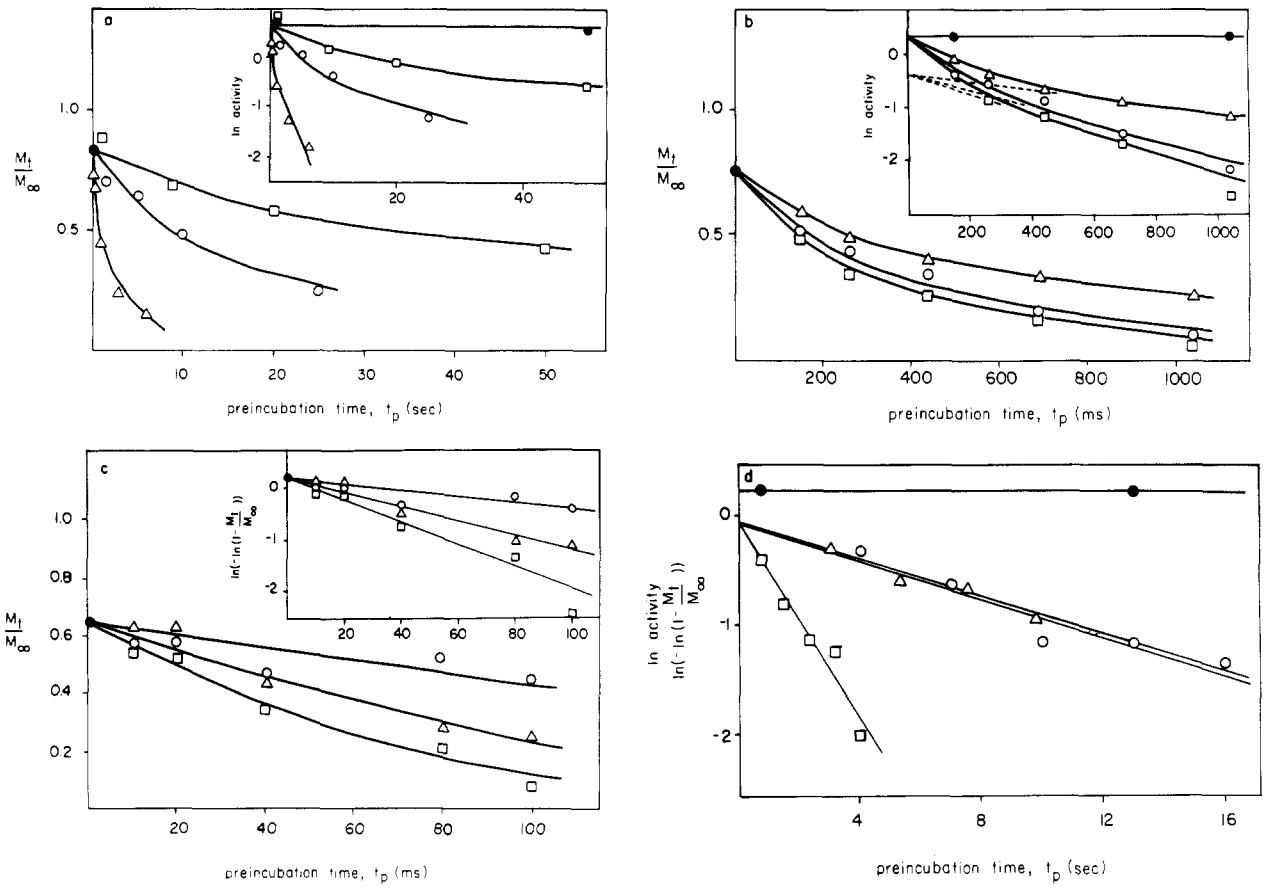

FIGURE 1: Desensitization of the receptors with varying GABA concentration. Fractional equilibration of the $^{36}Cl^-$ influx, M_t/M_∞, was measured in a constant assay after various times of preincubation with GABA as described under Experimental Procedures. The fitted lines were computed from eq 4. The measurements made with no GABA in the preincubation (●) are independent of t_p and represent zero preincubation time. (a) Desensitization with low GABA concentrations: (□) 1.0 μM; $\alpha = 0.09\ s^{-1}$, $\beta = 0.007\ s^{-1}$; (○) 7.0 μM, $\alpha = 0.28\ s^{-1}$, $\beta = 0.035\ s^{-1}$; (△) 60 μM GABA, $\alpha = 3.8\ s^{-1}$, $\beta = 0.3\ s^{-1}$. (Inset) Semilog plot of the biphasic decrease of ion flux activity [$-\ln(1 - M_t/M_\infty)$]. The lines correspond to $J_{A(t_p=0)} = 14\ s^{-1}$, $J_{B(t_p=0)} = 3\ s^{-1}$, and the values of α and β given above. (b) Desensitization with higher concentrations: (△) 100 μM, $\alpha = 6.0\ s^{-1}$, $\beta = 0.7\ s^{-1}$; (○) 250 μM, $\alpha = 8.0\ s^{-1}$, $\beta = 1.5\ s^{-1}$; (□) 500 μM, $\alpha = 10.0$, $\beta = 1.8\ s^{-1}$. (Inset) Semilog plot of the biphasic decrease of ion flux activity. The lines correspond to $J_{A(t_p=0)} = 9.8\ s^{-1}$ and $J_{B(t_p=0)} = 2.4\ s^{-1}$. (c) Desensitization in the faster phase in a short time period: (○) 40 μM, $\alpha = 5\ s^{-1}$; (△) 100 μM, $\alpha = 12\ s^{-1}$; (□) 500 μM, $\alpha = 22\ s^{-1}$. (Inset) Semilog plot of the first-order decrease of ion flux activity. Less than 5% of the activity of this preparation decreased in the slower phase. (d) Desensitization in the slower phase: (△) 40 μM, $\beta = 0.085\ s^{-1}$; (□) 100 μM, $\beta = 0.44\ s^{-1}$; (○) 1000 μM (time scale is decreased 10-fold), $\beta = 0.86\ s^{-1}$. Semilog plot of the first-order decrease of ion flux activity. In this experiment the fast phase is completed before the first measurements; $J_{A(t_p=0)} = 4.0\ s^{-1}$ and $J_{B(t_p=0)} = 3.3\ s^{-1}$. The different concentrations extrapolate to the same ordinate intercept ($t_p = 0$), $I_B = -0.073 \pm 0.020$.

intercepts are constant for given assay conditions. On the other hand if the second phase of desensitization were of influx due to the remaining activity of the same receptor after its desensitization in the fast phase to a preequilibrium value, the extrapolated intercept of the slower phase would be given by eq 9, where K_p is the GABA concentration dependent equi-

$$I_B = \ln\left(\frac{J_{A(t_p=0)}}{1 + 1/K_p}\,\frac{1 - e^{-\beta t}}{\beta}\right) \qquad (9)$$

librium constant for the faster desensitization process, in the preincubation conditions. That is, $K_p = \sum[A]/\sum[D]$, the ratio of the total concentration of receptor in the active state to that in the desensitized state due to the faster process. In this case this intercept would decrease with increasing GABA concentration (decreasing K_p) in the preincubation.

The experimental data were fitted to plots of M_t/M_∞ against t_p or of ln [$-$ ln $(1 - M_t/M_\infty)$] against t_p computed from eq 4–6. From experiments in the appropriate time regions values for α_p and β_p and $I_{(A+B)}$ and I_B were obtained.

RESULTS

The specific, GABA-mediated $^{36}Cl^-$ uptake by the membrane preparation was reduced by preincubation with a solution containing GABA. This loss of chloride flux activity increased with increasing preincubation time. The decrease in $^{36}Cl^-$ influx in a constant subsequent incubation was used to follow the processes of desensitization of GABA receptor. Figure 1 shows typical measurements of the decrease in the ion flux with increasing preincubation time over a range of GABA concentrations. The decrease in ion flux activity, ln [$-$ ln $(1 - M_t/M_\infty)$], was nonlinear with low (Figure 1a) and high (Figure 1b) GABA concentrations. By taking the measurements over the appropriate time regions (Figure 1c,d), the desensitization was shown to be biphasic consisting of a faster and a slower process each kinetically first order. The faster process was completed in ca. 50 s with 1 μM GABA (Figure 1a) and ca. 200 ms with 500 μM GABA (Figure 1b). The remaining activity decreased at a rate 10–16-fold more slowly.

In first-order plots of measurements made in a time range longer than that of the faster phase the extrapolated ordinate intercept of the slower phase was independent of GABA concentration (Figure 1c) when the faster phase was present.

The rates of both the faster and the slower phases increased with increasing GABA concentration. In certain conditions the plot of ln (activity) against preincubation time was linear, demonstrating the first-order kinetics of desensitization. For

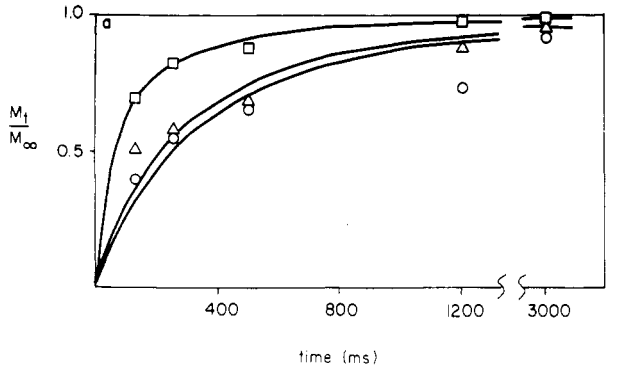

FIGURE 2: Progress of GABA-mediated $^{36}Cl^-$ uptake measured as described in the accompanying paper (Cash & Subbarao, 1987a). M_t/M_∞ is the fractional equilibration of ion flux. $^{36}Cl^-$ uptake with 1000 μM GABA (the desensitization assay conditions): (□) with no preincubation; (△) with 200-ms preincubation with 1000 μM GABA; (○) with 300-ms preincubation with 1000 μM GABA. The lines were computed with eq 1, 5, and 6 with $J_{A(t_p=0)} = 11.1\ s^{-1}$, $\alpha = 12\ s^{-1}$, $J_{B(t_p=0)} = 3.8\ s^{-1}$, $\beta = 0.8\ s^{-1}$. The value of M_∞ was the same (2082 counts) for all these experiments.

example, the slower phase was seen to be linear ($t_p \gg 1/\alpha$) over the loss of 80% of the activity (Figure 1c), and the faster phase was seen to be linear ($Be^{-\beta_p t_p} \ll Ae^{-\alpha_p t_p}$) over the loss of 80% of the activity (Figure 1d). Values for α_p and β_p were determined with GABA concentrations ranging over a 10000-fold concentration range (Figure 3).

The progress of the chloride influx in the assay (second) incubation is shown in Figure 2. In addition, this shows the influx curves after preincubation with saturating GABA concentration for 200 and 300 ms. The influx with no preincubation is biphasic with a faster phase which alone would account for 60% of the flux equilibration. After preincubation the fast phase is substantially removed, and over 90% of the influx is completed in a single slower phase. The activity loss between 200-ms and 300-ms preincubation is much slower than that in the first 200 ms. It is notable that with or without preincubation the chloride influx approaches the same final value, which corresponds to equilibration of ion flux with complete chloride exchange into the specifically, GABA receptor accessed, internal volume, 0.19 μL/mg of protein in this experiment.

In control experiments to investigate the possibility of instability of the GABA concentration in these experiments (Cash & Subbarao, 1987a), the GABA-mediated chloride influx was shown to be unaffected by the presence of known inhibitors of the GABA uptake process, indicating that GABA uptake was not significant during these experiments. Second, the unspecific chloride influx was unaffected by known GABA receptor antagonists indicating that chloride influx due to endogenous GABA was not significant in these experiments.

From the measured ordinate intercepts of the fast phase [$I_{(A+B)}$] and the slow phase (I_B) of desensitization (eq 7 and 8) and the values of α and β in the second ($^{36}Cl^-$ uptake) incubation, the values of $J_{A(t_p=0)}$ and $J_{B(t_p=0)}$ were determined. These are a measure of the ion flux activities, which decay in the two phases with the rate constants α and β, respectively. Generally the initial J_A value was about 4-fold larger than the initial J_B value, but in occasional experiments practically all the activity decrease (desensitization) was in either the faster phase or the slower phase. Of 17 experiments in which both intercepts were determined, the fraction of activity decaying in the slow phase [$J_B/(J_A + J_B)$] was 0.16 ± 0.04 (13 experiments), and 4 experiments gave extreme values of <0.05, 0.06, 0.41, and >0.95.

Variations of the measured values, α and β, with different rat brain preparations ($\sigma = \pm\lesssim 30\%$) were greater than the experimental error as evidenced by different experiments with the same preparation ($\sigma = \pm\lesssim 10\%$).

Discussion

Neurotransmitter receptor mediated transmembrane flux of a specific ion in a suspension of membrane vesicles was first demonstrated with acetylcholine receptor from electric fish (Kasai & Changeux, 1971). Since then techniques have been developed to use this tool to study the function and mechanism of channel-forming receptors (Hess et al., 1983; Cash et al., 1985; Udgaonkar & Hess, 1986). Rapid-mixing flow techniques with membrane vesicles enable one to measure specifically the transmembrane transport of a single ionic species with constant, defined solution concentrations, allowing resolution of the ion flux and the desensitization processes which together control the extent of the transmembrane ion flux. These measurements can be made with low concentrations of receptor in membrane prepared directly from the organ with a minimum of artifactual modification.

In these measurements of the desensitization of GABA receptor from rat brain (Figure 1), the conditions of $^{36}Cl^-$ influx into the vesicles do not allow complete equilibration of isotope exchange. Therefore a decrease of ion flux activity due to preincubation results in a decrease of $^{36}Cl^-$ uptake by the vesicles. The ion flux activity, which is a measure of the open-channel state of the receptors, can be followed quantitatively (eq 4).

The desensitization was seen to be biphasic (Figure 1a,b) and was shown to be made up of two distinct exponential decays of activity (Figure 1c,d). Since these processes differed in rate by at least 10-fold, they could be readily resolved, and their rates were measured over a 10000-fold concentration range of GABA.

These two phases of desensitization might have represented different receptors or two phases of desensitization of the same receptor. These two possibilities can be distinguished. In the latter case the ion flux activity decreasing in the second phase would initially be approximately that due to the preequilibrium mixture of active and desensitized receptors at the end of the first phase. Below saturation with GABA, this equilibrium value would be dependent on GABA concentration, so the flux activities decaying in the two phases would vary with GABA concentration and the intercept of the semilog plots in Figure 1 would be given by eq 9. On the other hand if the flux activities are due to different receptors, the intercept of these plots will be independent of GABA concentration and given by eq 8. In a preparation that showed a fast drop of activity in a fast phase with 40 μM GABA, the ordinate intercept remained constant with increasing GABA concentration through a 10-fold increase in desensitization rate (Figure 1d). This diagnostic plot showed that the two observed phases of desensitization correspond to two distinguishable receptors that are not interconvertible during the experiment. These measurements of receptor function do not preclude that the two distinguishable receptors are different forms of the same protein having different rates of desensitization.

The two receptors might be separately situated on different membranes or both present on the same membrane. That is, in these experiments, we might be following chloride isotope exchange into two populations of internal volume (vesicles) each enclosed by a different membrane and accessed via a different receptor or into a single population of vesicles bounded by membrane containing both receptors. An examination of the time course of $^{36}Cl^-$ uptake in the assay incubation conditions (Figure 2) allows a distinction between these

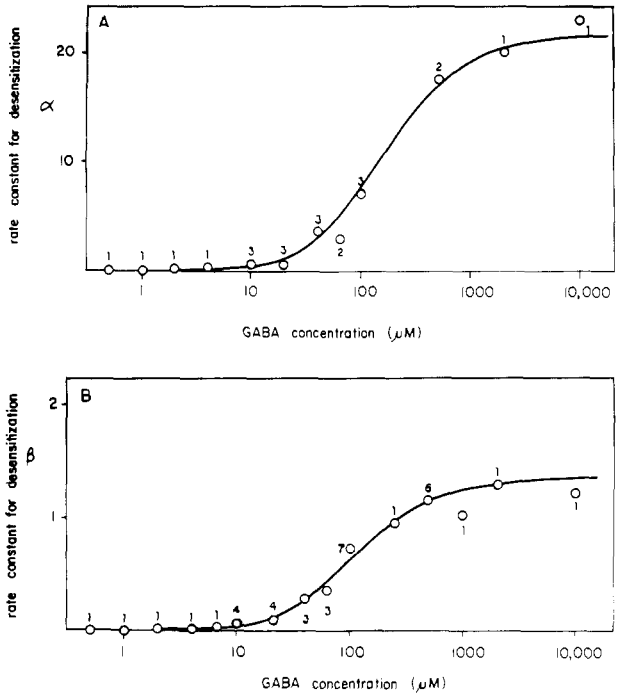

FIGURE 3: Dependence on GABA concentration of the rate constants for desensitization of (A) the faster desensitizing receptor, α, and (B) the slower desensitizing receptor, β. The points are means of the number of experiments indicated by the figure next to the point. The standard deviation for experiments with *different* preparations was ±20%. The lines are computed from the expression derived from the minimal kinetic model (Figure 4 and legend), using the values (A) $\alpha_{max} = 21.5\ s^{-1}$ and $K_A = 70\ \mu M$ and (B) $\beta_{max} = 1.35\ s^{-1}$ and $K_B = 49\ \mu M$.

possibilities. With no preincubation, $^{36}Cl^-$ exchange with 1000 μM GABA progresses to completion in two phases, with a fast phase, in which about 60% of the $^{36}Cl^-$ uptake occurs, terminated by the first desensitization. When the membrane is given a preincubation with 1000 μM GABA for 200 or 300 ms, the ion flux activity of the faster desensitizing receptor is decreased 11- and 37-fold, respectively, and this would reduce the chloride uptake in the faster phase to a small fraction (0.075 and 0.020, respectively), since the desensitization rates are unchanged in these constant conditions. After such preincubations with GABA, the fast phase of ion flux is seen to have disappeared, and the chloride influx proceeds to completion in the slower phase. The same final influx of $^{36}Cl^-$ was observed with or without preincubation, corresponding to equilibration of chloride exchange with the same internal volume. This failure to close a compartment of internal volume and reduce the chloride flux amplitude by preincubation showed that there is no significant internal volume compartment accessed only by the faster desensitizing receptor and that both receptors are on the same membrane bounding the vesicles.

The activities of the two receptors (J_A and J_B) could be estimated from the ordinate intercepts of the faster and the slower phases of desensitization by eq 7 and 8 (Figure 1). These varied with the rat brain preparations, which occasionally contained activity of only one of the types of receptor. To estimate the relative concentrations of the receptors, the relative values of their open-channel conductances or specific reaction rates of ion translocation ($\bar{J}$) (Hess et al., 1981) and their equilibria of channel opening (Φ) must be known. The concentration of each receptor (with respect to internal volume) R' is given by eq 10. If the rates of ion translocation

$$R' = J(1 + \Phi)/\bar{J} \qquad (10)$$

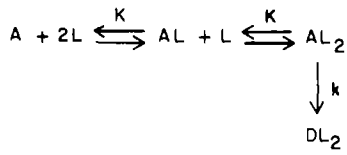

FIGURE 4: Minimal model relating GABA concentration to desensitization rate for the two receptors. An active state of the receptor, A, can bind two ligand molecules with microscopic dissociation constants, K, to give a doubly liganded species which is converted (desensitized) with a rate constant, k, to an inactive state, D, which does not form open channels. The rates of desensitization are given by the expression α or $\beta = k/(1 + K/[L])^2$, where [L] is the ligand concentration and k is the maximum rate of desensitization. The reversal of desensitization or the binding equilibria of the desensitized species or the formation of additional states of the receptor is not indicated merely because the results reported herein do not give information on them.

and the channel-opening equilibria are approximately the same for the two receptors, the concentration of the faster desensitizing receptor is, on average, about 5 times larger than that of the slower desensitizing receptor. Apparently the faster desensitizing receptor is the predominant one.

The dependencies of the rate coefficients α and β on GABA concentration are shown in Figure 3. The steepness of these response curves precludes the measured desensitization processes resulting from the binding of one GABA molecule per receptor. The simplest scheme that explains the data in this paper, shown in Figure 4, involves two binding sites for GABA, both of which must be occupied for desensitization. Desensitization of the receptors with only one GABA molecule bound to these two sites is not indicated by these results alone and can account for no more than 5% of the maximal response. However, these data do not rule out a relatively very low rate of desensitization with only one GABA binding site occupied, and this possibility was supported by a comparison of the desensitization rates with the rates of GABA-mediated chloride ion flux (Cash & Subbarao, 1987a).

The dependence of desensitization rate on GABA concentration is strikingly similar for the two receptors. The half-response concentrations are 150 μM for α and 114 μM for β. The faster desensitizing receptor has a 16-fold greater maximum desensitization rate. The relationship between the two receptors remains speculative. We should not completely rule out artifactual modification of one receptor to the other, but several protease inhibitors were added to the preparation, which was kept at 0–4 °C, and no interconversion was detected in several hours during the experiments. Other possibilities are that they are different forms of the same protein, not interconverted in the experiments, differently, in vivo modulated forms of the same receptor or different posttranslational modifications, synaptic and nonsynaptic receptor, or completely different proteins. Distinguishable GABA receptors on neurones have been reported on the basis of electrophysiological experiments (Alger & Nicoll, 1982; Dunlap & Fischbach, 1981; Bowery et al., 1980) and high-affinity ligand binding experiments (Bowery, 1983; Enna & Gallagher, 1983; Tallman & Gallager, 1985; Guidotti et al., 1979), but these might involve channels of different ionic specificity. Our results show that there are distinguishable receptors that form chloride channels.

The desensitization rates reported here are faster than previously reported for GABA receptor. The maximal rate constants are 22 s^{-1} ($t_{1/2}$ = 32 ms) and 1.3 s^{-1} ($t_{1/2}$ = 0.5 s). Observation of these two fast phases does not preclude the occurrence of additional slower phases of desensitization in this preparation depleting the activity of a remaining small fraction of the activity. In physiological studies, GABA re-

ceptor responses from various sources have desensitized in a few seconds (Numann & Wong, 1984; Thalman & Hershkowitz, 1985; Gallagher et al., 1983; Ozawa & Yuzaki, 1984; Akaiki et al., 1985), tens of seconds (Hackman et al., 1982; Adams & Brown, 1975), or minutes (Sarne, 1976; Epstein & Grundfest, 1970; Dudel & Hatt, 1976). In several studies the loss of response was seen to be exponential (Hackman et al., 1982; Numann & Wong, 1984; Thalman & Hershkowitz, 1985; Gallagher et al., 1983). As this work was nearing completion, desensitization of GABA receptor in a rat brain synaptoneurosome preparation by muscimol was reported (Schwartz et al., 1986a,b).

The mean desensitization rate of the predominant receptor with two bound GABA molecules (α = 22 s^{-1}) corresponds to a mean lifetime of the active receptor of 47 ms. This is only slightly larger than reported values for the mean channel lifetime of the GABA receptor calculated from single-channel measurements or noise analysis. A value of 25 ms was reported for chick brain GABA receptor expressed in oocytes (Miledi et al., 1982), and similar values of ca. 30 ms or more were reported in spinal chord (Barker & Mathews, 1981; Barker et al., 1982; Hamill et al., 1983) and lamprey CNS (Gold & Martin, 1982). The similarity of the fast-phase desensitization rates reported here with these electrophysiological measurements made with different techniques and different preparations is striking. The fast desensitization process is not much slower than the reported channel closing. In single-channel measurements, a burst of current through a receptor channel flickers due to several very brief interruptions (Sakmann et al., 1983). The end of the burst has been attributed to dissociation of ligand and channel closing while the interruptions have been attributed to channel closures without dissociation of ligand (Colquhoun & Sakmann, 1981), which have been suggested to be different mechanisms of channel closing (Sakmann et al., 1983). If the generally found value for the lifetime of the GABA receptor channel applies to the predominant, faster desensitizing receptor under study here, rapid desensitization must be considered as a possible mechanism of termination of the current burst in this case.

The physiological role of desensitization is of great interest. Desensitization can modify the electrical response due to channel opening. The high rates of desensitization reported here could take effect during a relatively short signal as well as with repetitive action at the synapse (Krnjevik, 1981). The desensitization responses of the different receptors on the same membrane, with the predominant receptor desensitizing 10–16 times faster than a minor one, may play a significant role. The duration of postsynaptic current in voltage clamp experiments has varied with the situation from short times to a half decay time of as large as 100 ms (Dingledine & Korn, 1985). In this instance ca. 80% of the predominant receptor and ca. 20% of the minor receptor would be desensitized during the signal.

The GABA receptor is the second receptor to be studied by these techniques. The methods developed with the acetylcholine receptor from electric fish (Hess et al., 1983; Cash et al., 1985; Cash & Hess, 1981; Aoshima et al., 1981) may have general applicability for the study of channel-forming receptors in the brain.

ACKNOWLEDGMENTS

We thank Dr. R. M. Langer for his work with the computing software.

Registry No. GABA, 56-12-2; Cl^-, 16887-00-6.

REFERENCES

Adams, P. R., & Brown, D. A. (1975) *J. Physiol. (London) 250*, 85–120.
Akaiki, N., Hattori, K., Imomata, N., & Oomura, Y. (1985) *J. Physiol. (London) 360*, 367–386.
Alger, B. E., & Nicoll, R. A. (1982) *J. Physiol. (London) 328*, 125–141.
Allan, A. M., Harris, R. A., Subbarao, K., & Cash, D. J. (1985) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 44*, 634.
Aoshima, H., Cash, D. J., & Hess, G. P. (1980) *Biochem. Biophys. Res. Commun. 92*, 896–904.
Aoshima, H., Cash, D. J., & Hess, G. P. (1981) *Biochemistry 20*, 3467–3474.
Barker, J. L., & Mathews, D. A. (1981) *Science (Washington, D.C.) 212*, 358–361.
Barker, J. L., McBurney, R. N., & MacDonald, J. F. (1982) *J. Physiol. (London) 322*, 365–387.
Bowery, N. G. (1983) in *The GABA Receptors* (Enna, S. J., Ed.) pp 177–213, Humana, Clifton, NJ.
Bowery, N. G., Hill, D. R., Hudson, A. L., Doble, A., Middlemiss, D. N., Shaw, J., & Turnbull, M. (1980) *Nature (London) 283*, 92–94.
Cash, D. J., & Hess, G. P. (1981) *Anal. Biochem. 112*, 39–51.
Cash, D. J., & Subbarao, K. (1987a) *Biochemistry* (following paper in this issue).
Cash, D. J., & Subbarao, K. (1987b) *FEBS Lett. 217*, 129–133.
Cash, D. J., Aoshima, H., Pasquale, E. G., & Hess, G. P. (1985) *Rev. Physiol., Biochem. Pharmacol. 102*, 73–117.
Colquhoun, D., & Sakmann, B. (1981) *Nature (London) 294*, 464.
Dingledine, R., & Korn, S. J. (1985) *J. Physiol. (London) 366*, 387–409.
Dudel, J., & Hatt, H. (1976) *Pfluegers Arch. 364*, 217–222.
Dunlap, K., & Fischbach, G. D. (1981) *J. Physiol. (London) 317*, 519–535.
Enna, S. J., & Gallagher, J. P. (1983) *Int. Rev. Neurobiol. 24*, 181–212.
Epstein, R., & Grundfest, H. (1970) *J. Gen. Physiol. 56*, 33–45.
Gallagher, J. P., Nakamura, J., & Shinnick-Gallagher, P. (1983) *J. Pharmacol. Exp. Ther. 266*, 876–884.
Gold, M. R., & Martin, A. R. (1982) *Nature (London) 299*, 828–830.
Guidotti, A., Gale, K., Suria, A., & Toffano, G. (1979) *Brain Res. 172*, 566–571.
Hackman, J. C., Auslander, D., Grayson, V., & Davidoff, R. A. (1982) *Brain Res. 253*, 143–152.
Hamill, O. P., Bormann, J., & Sakmann, B. (1983) *Nature (London) 305*, 805–808.
Harris, R. A., & Allan, A. M. (1985) *Science (Washington, D.C.) 228*, 1108–1110.
Hess, G. P., Cash, D. J., & Aoshima, H. (1979) *Nature (London) 282*, 329–331.
Hess, G. P., Aoshima, H., Cash, D. J., & Lenchitz, B. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 1361–1365.
Hess, G. P., Cash, D. J., & Aoshima, H. (1983) *Annu. Rev. Biophys. Bioeng. 12*, 443–473.
Houamed, K. M., Bilbe, G., Smart, T. G., Constanti, A., Brown, D. A., Barnard, E. A., & Richards, B. M. (1984) *Nature (London) 310*, 318–321.
Kasai, M., & Changeux, J. P. (1971) *J. Membr. Biol. 6*, 1–23, 24–57, 58–80.
Krnjevic, K. (1974) *Physiol. Rev. 54*, 518–540.

Krnjevic, K. (1981) in *GABA and Benzodiazepine Receptors* (Costa, E., DiChara, G., & Gessa, G. L., Eds.) pp 111–120, Raven, New York.
Miledi, R., Parker, I., & Sumikawa, K. (1982) *Proc. R. Soc. London, B 216*, 509–515.
Numann, R. E., & Wong, R. S. (1984) *Neurosci. Lett. 47*, 289–294.
Ozawa, S., & Yuzaki, M. (1984) *Neurosci. Res. (Shannon, Irel.) 1*, 275–293.
Parker, I., Gundersen, C. B., & Miledi, R. (1986) *J. Neurosci. 6*, 2290–2297.
Sakmann, B., Bormann, J., & Hamill, O. P. (1983) *Cold Spring Harbor Symp. Quant. Biol. 48*, 247–257.
Sânchez, G. M., Toledo, M. C., & Gonzâlez, M. P. (1984) *Rev. Esp. Fisiol. 40*, 375–380.
Sarne, Y. (1976) *J. Physiol. (London) 257*, 779–790.
Schwartz, R. D., Skolnick, P., Seale, T. W., & Paul, S. M. (1986a) *Adv. Biochem. Pharmacol. 41*, 33–49.
Schwartz, R. D., Suzdak, P. D., & Paul, S. M. (1986b) *Mol. Pharmacol. 30*, 419–429.
Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., & Klenk, D. C. (1985) *Anal. Biochem. 150*, 76–85.
Subbarao, K., & Cash, D. J. (1985) *Soc. Neurosci. Abstr. 11*, 275.
Tallman, J. F., & Gallager, D. W. (1985) *Annu. Rev. Neurosci. 8*, 21–44.
Thalman, R. H., & Hershkowitz, N. (1985) *Brain Res. 342*, 219–233.
Thampy, K. G., & Barnes, E. M. (1984) *J. Biol. Chem. 259*, 1753–1757.
Ticku, M. K., & Olsen, R. W. (1977) *Biochim. Biophys. Acta 464*, 519–529.
Udgaonkar, J. B., & Hess, G. P. (1986) *J. Membr. Biol. 93*, 93–109.
Wong, E. F. H., Leeb-Lundberg, L. M. F., Teichberg, V. I., & Olsen, R. W. (1984) *Brain Res. 303*, 267.

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

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Received May 12, 1987; Revised Manuscript Received August 11, 1987

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

† This work was supported in part by a grant from the Research Council of the University of Missouri—Columbia Medical School.
‡ Recipient of a Missouri Institute of Psychiatry fellowship.

0006-2960/87/0426-7562$01.50/0