Volume 217, number 1, 129-133 FEB 04793 June 1987

Two desensitization processes of GABA receptor from rat brain

Rapid measurements of chloride ion flux using quench-flow techniques

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Received 26 March 1987

Two rapid phases of GABA receptor desensitization, which proceeded with a 10-fold difference in rates, were detected in two types of experiment with membrane vesicle preparations from rat cerebral cortex. (i) The time course of GABA-mediated $^{36}Cl^-$ influx progressed in two phases. (ii) The $^{36}Cl^-$ influx was decreased, by preincubation with GABA, in two phases. Measurements were made in the time range 10–1000 ms. The major loss of channel opening activity occurred in the faster phase, which was complete in 100 ms with saturating GABA concentrations. The remaining activity decreased in a slower phase in a few seconds with a 10-fold slower rate. The faster phase of desensitization was more than 10-fold faster than previously observed and the slower phase was slightly faster than previously reported measurements with GABA receptor. Both desensitization processes had a similar dependence on GABA concentration with a half response at $\sim 100\ \mu M$ GABA.

GABA receptor; Desensitization; Cl^- uptake; Ion flux; Quench flow; (Rat brain)

1. INTRODUCTION

A number of channel-forming neurotransmitter receptors are desensitized by their neurotransmitter. During exposure to the neurotransmitter the receptor is transformed to a state which does not form open channels. This phenomenon, which is not well understood, may be of physiological importance as well as of interest with regard to biochemical mechanism. Desensitization of γ-aminobutyric acid (GABA) receptors from crustacean muscle [1,2], hippocampal neurones [3–5] and ganglion cells [6–9] has been observed and a single phase of desensitization in the time range of several seconds has been reported.

Desensitization of acetylcholine receptor from electric fish has been studied by measuring the acetylcholine-mediated cation flux, with membrane preparations containing vesicles, using rapid-mixing kinetic techniques [10,11]. Such studies require a suspension of membrane vesicles containing active neurotransmitter receptor, which mediates transmembrane ion flux. Transmembrane Cl^- flux mediated by GABA receptor has been demonstrated with crayfish muscle strips [12], rat brain slices [13] and cultured neurones from chick brain [14]. Recently membrane preparations from rat or mouse brain homogenates have been shown to exhibit GABA-mediated Cl^- flux [15–19]. The pharmacology of this specific

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Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50

Cl^- flux [18] indicated that the receptor was of the $GABA_A$ type [20–22].

Here, we report that GABA receptor on native membrane vesicles from rat brain is desensitized much more rapidly than in previous observations, and that the desensitization occurs in two distinct phases. Recognition of two desensitization processes is relevant to the interpretation of pharmacological measurements of Cl^- flux as well as to the mechanisms of neuronal signal transmission.

2. MATERIALS AND METHODS

Male Sprague-Dawley rats, 4–6 weeks old, were killed by decapitation. All manipulations were performed on ice and solutions were at 0–4°C. The cerebral cortex was sliced and homogenized in 0.32 M sucrose, 10 mM Hepes, pH 7.5 (solution A) (30 ml) with a Virtis homogenizer 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 and the mixture was centrifuged for 4 min at $270 \times g$. The supernatant was centrifuged for 30 min at $23\,640 \times g$. The pellet was resuspended in 8 ml solution B using a glass-teflon hand homogenizer, layered on a 4–12% Ficoll gradient in solution A and centrifuged for 1 h at $110\,000 \times g$. The middle band (9–10% Ficoll) was diluted with 2 vols solution B and centrifuged for 30 min at $35\,000 \times g$. The pellets were resuspended in solution B and adjusted to 750 μg protein/ml. The experiments described gave similar results when the Ficoll gradient separation was omitted.

Quench-flow experiments were performed as described in [23,24]. The quench-flow machine was thermostatted at 30.0°C and the reagent solutions were preequilibrated at 30°C. The membrane vesicle preparation was warmed to 30°C on loading into the machine syringe. This thermal equilibration was completed within 2 min and the preparation was allowed to stand for a further 1 min before actuation. Experiments were at 30°C and pH 7.5. The addition of known inhibitors of the sodium-dependent GABA uptake, nipecotic acid (1 mM) and guvacine (1 mM), made no difference to the ion flux measurements with a concentration as low as 10 μM GABA in the time region of these experiments. $^{36}Cl^-$ was obtained from ICN Radiochemicals (Richmond, CA).

2.1. *Time course of Cl^- flux*

The ion flux reaction was initiated (receptor channels opened) by mixing the membrane suspension (225 μl) with an equal volume of solution B containing B and $^{36}Cl^-$ (20 μCi/ml), and quenched (channels closed) by mixing with the same volume of either (i) a solution of bicuculline (600 μM) kept at pH 3.0, with no added buffer (solution B without Hepes) (which reduces the pH of the reaction mixture insignificantly), or (ii) a solution of bicuculline methiodide (3000 μM) in solution B.

The quenched reaction mixture was passed immediately through a filter disc (Whatman glass fiber GF/C). The vesicles retained on the filter disc were washed with solution B, dried and counted with toluene-based scintillation fluid. Unspecific flux was determined in the same way but in the absence of GABA and was subtracted from the total flux to give the GABA-mediated, specific flux. At equilibration of the GABA-mediated flux the specific measurement was typically 2000–3000 counts on a background of ~1000 counts. Each determination was done in triplicate. M_∞ was determined by allowing an incubation time of 6 s with 1000 μM GABA.

2.2. *Time course of desensitization*

The membrane suspension was mixed with 1 vol. of a solution containing GABA. After the preincubation time this mixture was mixed with 1 vol. of a solution containing 3 mM GABA and $^{36}Cl^-$, and the Cl^- influx in 320 ms was measured in a second incubation as described above. In control experiments to check vesicle integrity during the experiment, GABA was omitted from the preincubation at the shortest and longest preincubation times. M_∞ was determined by omitting GABA from the preincubation and extending the second incubation time to 6 s. The unspecific flux baseline was determined by omitting GABA from both incubations.

2.3. *Treatment of data*

The concentration of open channel of receptor is reduced in two phases of desensitization with the first-order rate constants α and β. Uptake of the $^{36}Cl^-$ tracer, by isotope exchange through the open channel, occurs with the initial first-order flux rate constants J_A and J_B for the fast and slow phases, respectively. The $^{36}Cl^-$ uptake is given by eqn 1. In

the preincubation, the ion flux rate constants, proportional to the open channel concentration, are decreased by desensitization with the rate constants α_p and β_p (eqns 2,3). Combining eqns 1-3 gives eqn 4, which describes the decrease in $^{36}Cl^-$ uptake due to preincubation with GABA. The amplitudes of the two phases are given by A and B (eqns 5,6) which are constant since the second (assay) incubation has constant conditions and incubation time.

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

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

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

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

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

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

3. RESULTS AND DISCUSSION

Desensitization of the GABA receptor during a preincubation of the membrane preparation with GABA was followed by measuring the decrease in Cl^- exchange in a subsequent incubation (figs 1A,2). when the desensitization was followed in the time range 150–1000 ms it followed a single exponential decay of activity over at least an 80% drop in ion-influx activity (fig.1B). However, the fitted straight lines did not extrapolate to the initial activity at zero preincubation time, but the ordinate intercept corresponded to prior inhibition of 40% of the Cl^- influx. This suggested that the desensitization being followed is preceded by a much faster desensitization process. This faster desensitization process could be followed in the time range 10–100 ms (fig.2). A rapid desensitization which was kinetically first order was seen decreasing the Cl^- uptake by ~60%. This was complete before the slower phase was significant. Although desensitization rates of this receptor in different membrane preparations can vary by as much as 2-fold, these results indicate that there are

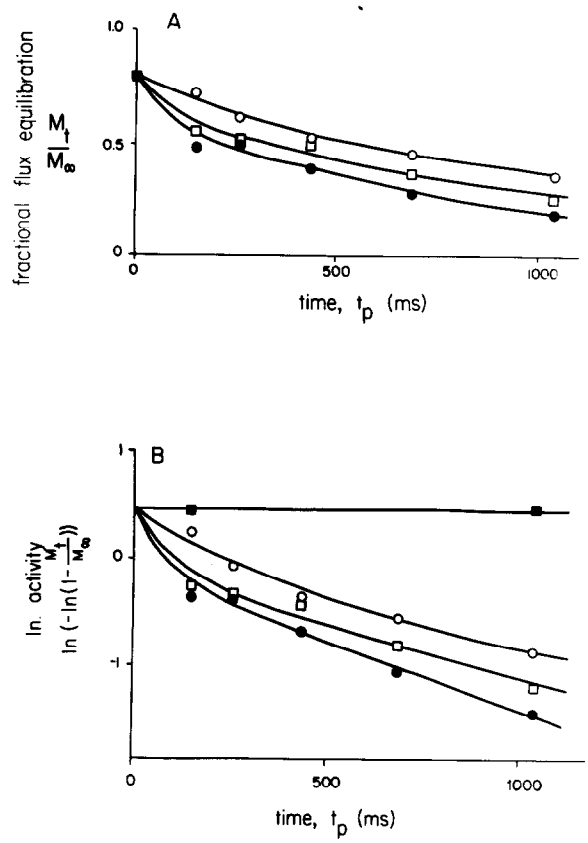

Fig.1. Desensitization of the receptor with GABA at 30°C and pH 7.5; 100 μM (○), 500 μM (□) and 2000 μM (●) GABA. M_t/M_∞ is the fractional equilibration of Cl^- influx; M_t denotes the counts in the specific vesicles; M_∞ denotes that at equilibration of Cl^- exchange into the specific vesicles. (A) Decrease of $^{36}Cl^-$ uptake in a constant assay after preincubation of the membrane with GABA for time t_p. The zero time t_p point (■) was obtained with no GABA in the preincubation, and this value was independent of t_p. (B) First-order plots of the data in (A) according to eqn 4 (if α and t are relatively large, A is constant for a given GABA concentration). The desensitization of receptor activity is biphasic. The control experiment with zero GABA concentration (■) is shown. The lines were calculated with values of $\alpha = 4.5$ s^{-1}, $\beta = 0.7$ s^{-1} (100 μM), $\alpha = 12$ s^{-1}, $B = 1.0$ s^{-1}, (500 μM) and $\alpha = 15$ s^{-1}, $\beta = 1.3$ s^{-1} (2000 μM GABA).

two rapid phases of receptor desensitization in this preparation, differing in rates by a factor of ten.

The existence of a rapid desensitization process leaving remaining receptor activity which is depleted in a slower desensitization process was

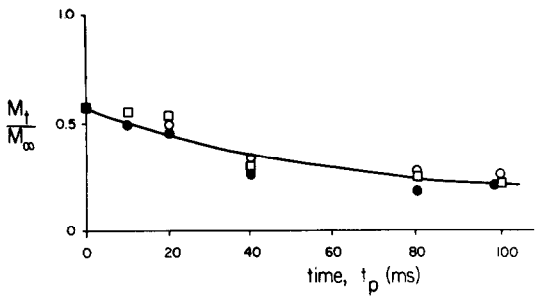

Fig.2. Desensitization of the receptor with GABA at 30°C and pH 7.5. Decrease of $^{36}Cl^-$ uptake in a constant assay incubation after preincubation of the membrane with GABA for time t_p. The GABA concentrations were 500 μM (○), 1000 μM (□) and 10000 μM (●). The zero time t_p point (■) was obtained with no GABA in the preincubation. The line, calculated from eqn 4, corresponds to 66% of the desensitization occurring in a fast phase with $\alpha_p = 20\ s^{-1}$ and $\beta_p \ll \alpha_p$.

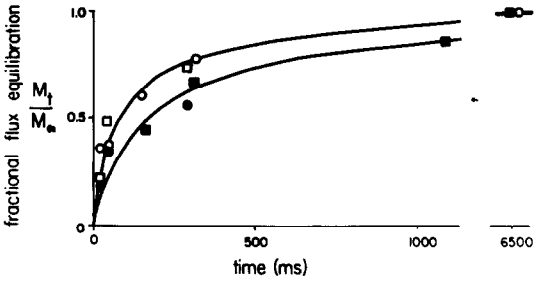

Fig.3. Biphasic uptake of $^{35}Cl^-$ mediated by 100 μM (filled symbols) and 500 μM (open symbols) GABA at 30°C and pH 7.5. M_t denotes the counts in the specific vesicles at the time t on the abscissa: M_∞ denotes that at equilibrium. The lines are calculated from eqn 1 with, for 100 μM, $J_A = 4.6\ s^{-1}$, $\alpha = 7.0\ s^{-1}$, $J_B = 0.4\ s^{-1}$, $\beta = 0.4\ s^{-1}$ and for 500 μM, $J_A = 9\ s^{-1}$, $\alpha = 13\ s^{-1}$, $J_B = 3\ s^{-1}$ and $\beta = 1.0\ s^{-1}$.

supported by experiments following the time course of the Cl^- exchange. The progress of $^{35}Cl^-$ uptake was much better fitted to a biphasic ion influx (eqn 1) than to a single phase [17]. The uptake of $^{36}Cl^-$ shown in fig.3 is biphasic with a rapid phase of ion flux followed by a slower phase. The two rates of desensitization required to fit these measurements are consistent with those determined by the preincubation method.

According to these data, the GABA receptor is almost saturated with 500 μM GABA and the half-response concentration is about 100 μM GABA. Both phases of desensitization have a similar dependence on GABA concentration. These initial responses of the receptor are associated with binding sites of lower affinity than those demonstrated by other studies [20–22]. A major origin of this discrepancy, in measurements made after some exposure of the receptor to GABA, is the presence of desensitized receptor which has an increased affinity for the ligand.

The occurrence of two different phases of desensitization giving rise to two phases of $^{36}Cl^-$ influx complicates the analysis of ion flux measurements. Measurements of the effects of varying conditions and of the presence of drugs made with GABA receptor should be treated cautiously since the relative contributions of the two phases of desensitization may not be consistent.

Two phases of desensitization have been seen in measurements of ion flux with acetylcholine receptors [25–27]. The fast phase of desensitization reported here is the fastest reported rate of desensitization of a receptor.

The 10-fold slower, second phase reported here is slightly faster than the fastest rates previously reported for GABA receptor [1–6].

ACKNOWLEDGEMENTS

This work was supported in part by a grant from the Research Council of the University of Missouri-Columbia Medical School. K.S. holds a Missouri Institute of Psychiatry fellowship. We thank Dr R.M. Langer for work with the computing software.

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