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Effect of a benzodiazepine (chlordiazepoxide) on a $GABA_A$ receptor from rat brain

Requirement of only one bound GABA molecule for channel opening

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Chlordiazepoxide (CDPX) enhanced the rate of chloride exchange mediated by the major $GABA_A$ receptor found on sealed native membrane vesicles from rat cerebral cortex. The initial rate constant for chloride exchange for this receptor, (J_A), a measure of open channel, was determined from the progress of GABA-mediated influx of $^{36}Cl^-$. The dependence of J_A on GABA concentration was hyperbolic in the presence of CDPX (150 μM, sufficient to give maximum enhancement of chloride exchange rate) but sigmoid in its absence. Enhancement of channel opening (10-fold at 0.3 μM GABA) decreased with increasing GABA concentration. The maximal response, above 1,000 μM GABA, was unaltered. The half-response concentration was reduced from 80 μM to 50 μM. CDPX alone caused no measurable $^{36}Cl^-$ exchange. In the presence of CDPX, channel opening occurred with only one bound GABA molecule, whereas in its absence, channel opening with two bound GABA molecules was much more favorable. This could not be direct allosteric modulation of the channel opening conformational change by binding of CDPX at effector sites, but could be explained by an additional change of the receptor on binding CDPX to give a closed state which gave channel opening mediated by a single GABA binding site. Another possibility is that CDPX could act at one of the channel opening binding sites without a postulated, second closed conformational state.

GABA receptor; Ion-channel; Quench flow; Benzodiazepine; Chlordiazepoxide; Chloride

1. INTRODUCTION

The mechanism by which $GABA_A$ receptor forms open channels on binding the neurotransmitter, γ-aminobutyrate (GABA) is of great interest regarding the mechanisms of protein transformations as well as the physiology of neurotransmission. Recent reviews have appeared [1–9]. GABA-Mediated channel opening is enhanced by anxiolytic drugs including the benzodiazepines [9–11] and barbiturates. Because $GABA_A$ receptors are now recognized to be members of a superfamily of homologous protein complexes, including nicotinic acetylcholine and glycine receptors, an appreciation of their mechanisms is relevant to channel forming neurotransmitter receptors in general. Furthermore, there may be differences in mechanism among different $GABA_A$ receptors containing different types or subtypes of subunit.

One approach to the study of function is the measurement of receptor-mediated chloride exchange using radioactive tracer ion ($^{36}Cl^-$). Using rapid mixing, quench-flow techniques, the function of *native* $GABA_A$ receptor in membrane prepared *directly* from brain could be investigated [12,13]. Different receptors on the same membrane, distinguishable by their desensitization rates, were observed by such measurements of *function* [14,15]. Using the same techniques, these receptors were found to be affected differently by pentobarbital [16]. Here we report the effect of a well-known benzodiazepine, chlordiazepoxide (CDPX, Librium), on the rate of chloride exchange mediated by the major receptor found on the sealed vesicles of native membrane from rat cerebral cortex. The mechanism is changed so that there is a hyperbolic dependence of channel opening on GABA concentration, extending the channel opening to lower concentrations.

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2. MATERIALS AND METHODS

Male Sprague–Dawley rats, 4–6-weeks old, were killed by decapitation. The cerebral cortex was rinsed with cold saline, cut into 1 mm slices and suspended in 30 ml solution A (0.32 M sucrose, 10 mM HEPES, pH 7.5) containing the protease inhibitors, phenylmethylsulfonyl fluoride (1 mM), aprotinin (10 μg/ml), antipain (5 μg/ml), leupeptin (5 μg/ml), pepstatin A (5 μg/ml), and the antioxidant, butylated hydroxytoluene (20 μM). All manipulations were performed at 0–4°C. The mixture was homogenized with a Virtis 45 homogenizer (setting 30, 5 s). An equal volume of solution B (145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.2 mM $CaCl_2$, 10 mM glucose, 10 mM HEPES, pH 7.5) was added and the mixture was centrifuged at 270 × g for 4 min. The supernatant was centrifuged at 6,500 × g for 20 min. The pellet was resuspended in 10 ml solution B using a glass–Teflon hand ho-

mogenizer, and 15 ml solution B was added before centrifuging at 4,000 × *g* for 15 min. The pellet was resuspended in solution B and adjusted to 750 μg protein/ml.

2.1. *Progress of $^{36}Cl^-$ influx*

Rapid mixing and short reaction times were achieved by the quench–flow technique [17,18] in continuous flow or pulsed mode [19] with an in-line filter disc assay [18]. The experiments were performed at 30°C. The membrane vesicle preparation was warmed to 30°C from 0°C within 2 min after loading into the machine and was held at 30°C for a further minute before actuation. The chloride flux was initiated (receptor channels opened) by mixing the membrane suspension (225 μl) with an equal volume of solution B containing $^{36}Cl^-$ (10 μCi/ml)(New England Nuclear) and the GABA and CDPX. The channels were closed by mixing with the same volume of solution B containing 3 mM bicuculline methiodide [20], and the mixture was rapidly passed through a glass fiber filter disc (Schleicher & Schuell Inc. No. 31) using a low vacuum (100 mm Hg below atmosphere). The vesicles, retained on the disc, were washed three times with solution B (10 ml), dried and counted with a scintillation cocktail for 10 min. The (−)bicuculline methiodide [21] was synthesized by methylation of (+)bicuculline (Sigma Chemical Co.) in methylene dichloride [15]. It has previously been demonstrated that this quenching of chloride flux is sufficiently rapid [15]. The background count, including non-specific influx, was 75 cpm at short times (<200 ms) rising to 270 cpm at 15 s, and was not decreased in the presence of 50 μM bicuculline methiodide. Typically, the maximal GABA-mediated influx was 150 cpm and the standard deviation was 6.7 cpm. For each point the GABA-mediated influx and the background were each determined in triplicate. In experiments with less than 10 μM GABA, the GABA uptake inhibitor, nipecotic acid (1 mM) was added to the solution.

3. RESULTS

The progress of GABA-mediated $^{36}Cl^-$ exchange into the sealed vesicles was followed after the simultaneous addition of GABA, the benzodiazepine (CDPX) and the $^{36}Cl^-$ isotope tracer. As previously described [15], the isotope uptake progressed in two phases, each terminated by desensitization. The $^{36}Cl^-$ influx can be described by equation 1, in which the initial first order rate

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

constants for chloride exchange, J_A, and J_B, for the fast and slow phases, are progressively attenuated by the desensitization processes with first order rate constants α and β, respectively. M_t/M_∞ is the fraction of equilibration of the isotope tracer in the reaction time, t, between the first mixing and the second mixing. These $^{36}Cl^-$ influx curves, exemplified in Fig. 1 were measured in the absence and presence of CDPX (150 μM) with GABA concentration varying over the whole concentration range of the response. The first phase was terminated by desensitization of the more rapidly desensitizing receptor at approximately half the total equilibration, within about 1 s (20 μM GABA), 2 s (10 μM), 6 s (3 μM), 15 s (1 μM) and 60 s (0.3 μM), respectively. The presence of CDPX without GABA did not cause detectable $^{36}Cl^-$ influx at concentrations up to 500 μM and times up to at least 15 s.

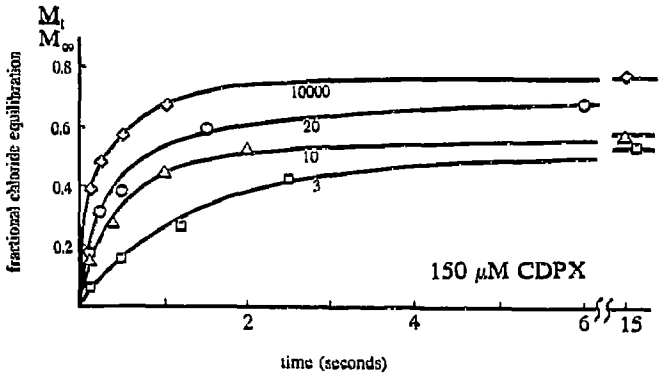


Fig. 1. Progress of chloride ion exchange mediated by GABA (3 μM (□), 10 μM (△), 20 μM (○), 1000 μM (◇) in the presence of CDPX (150 μM) at 30°C, pH 7.5. M_t/M_∞ is the fractional equilibration of $^{36}Cl^-$ influx; M_t denotes the counts in the specific vesicles; M_∞ denotes the counts at equilibration of Cl^- exchange into the specific vesicles. M_∞ was determined in the absence of CDPX with 1,000 μM GABA for 15 s and was 115 cpm. The ion flux proceeded in two phases described by equation 1 [15]. The lines are calculated with equation 1 with the following values for the parameters: 3 μM GABA, $J_A = 0.41$ s^{-1}, $\alpha = 0.60$ s^{-1}, $J_B = 0.01$ s^{-1}, $\beta = 0.08$ s^{-1}; 10 μM GABA, $J_A = 1.2$ s^{-1}, $\alpha = 1.8$ s^{-1}, $J_B = 0.05$ s^{-1}, $\beta = 0.2$ s^{-1}; 20 μM GABA, $J_A = 1.4$ s^{-1}, $\alpha = 3.0$ s^{-1}, $J_B = 0.47$ s^{-1}, $\beta = 0.65$ s^{-1}; 1,000 μM GABA, $J_A = 7.0$ s^{-1}, $\alpha = 19$ s^{-1}, $J_B = 1.3$ s^{-1}, $\beta = 1.2$ s^{-1}.

The initial rate constant for chloride exchange, J_A, determined by fitting equation 1 to the $^{36}Cl^-$ influx measurements as illustrated in Fig. 1, is shown as a function of GABA concentration in Fig. 2. This represents the initial rate without attenuation by desensitization. At the lower GABA concentrations (below 10 μM), the increase in chloride exchange rate and desensitization rate by CDPX was much greater for the first phase of chloride exchange (due to the faster desensitising receptor) than for the second phase, increasing the separation between the two phases and giving an improved precision of the determination of J_A. In the absence of CDPX the dependence of J_A on GABA concentration was sigmoid, in agreement with previously reported measurements under the same conditions [15]. In the presence of 150 μM CDPX this dependence was linear at low GABA concentrations (<10 μM) (Fig. 2B, Table I) corresponding to the foot of the hyperbolic

Table I

Enhancement of GABA-mediated chloride exchange rate by 150 μM chlordiazepoxide (CDPX)

GABA concentration (μM)	$^{36}Cl^-$ exchange rate constant $J_{A(CDPX)}$ (S^{-1}) ± 8%	Enhancement $J_{A(CDPX)}/J_A$
0.3	0.048	9.1
1.0	0.12	8.3
3.0	0.43	7.5
10.0	1.2	3.7
20.0	1.8	2.25

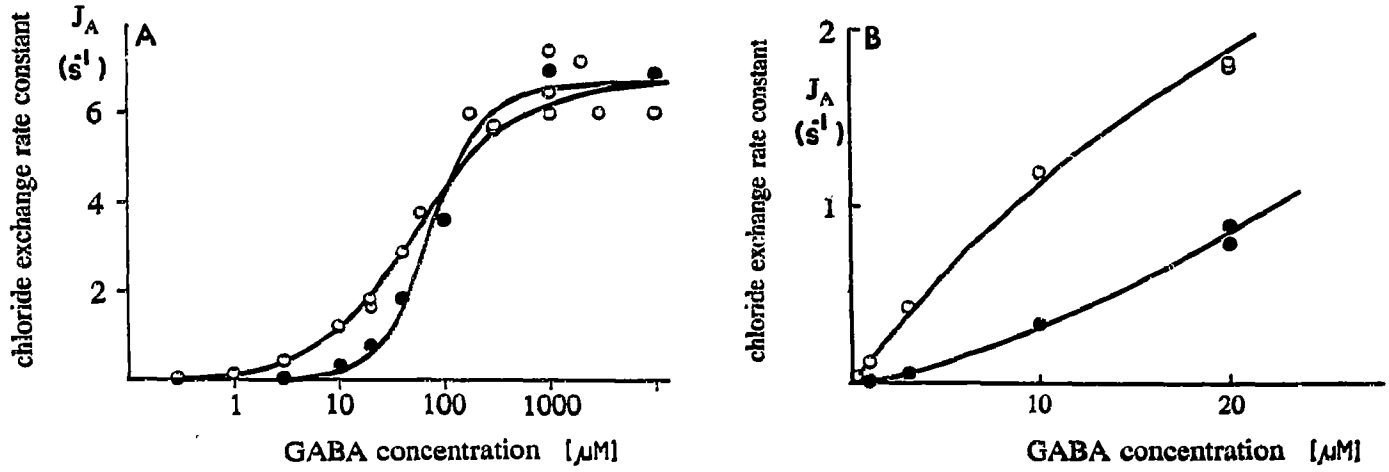


Fig. 2. Values of the initial first order rate constant for the first phase of chloride exchange, J_A, obtained by fitting equation 1 to the progress of $^{36}Cl^-$ influx as exemplified in Fig. 1. Measurements were made in the absence (●) and presence (○) of CDPX (150 μM). (A) On a logarithmic scale, the whole concentration range of response to GABA. The lines are calculated from the minimal kinetic scheme shown in Fig. 3B, with the values given in the text. (B) Plot showing the linear dependence (foot of the hyperbolic curve) of the initial exchange rate, J_A, on GABA concentration in the presence of CDPX with low GABA concentrations. The precisions of the determinations are within $\sigma = \pm 10\%$ with CDPX below 20 μM GABA, and within $\sigma = \pm 15\%$ elsewhere.

response. The enhancement due to CDPX decreased with increasing GABA concentration (Table I). At the maximum chloride exchange rate there was no enhancement (Fig. 2A). With CDPX the foot of the response curve was extended to lower GABA concentrations. The half-response GABA concentration was reduced by CDPX from about 80 μM to about 50 μM. This concentration of CDPX gives the maximal enhancement of J_A. With 10 μM GABA, the initial rate constant for chloride exchange, J_A, is increased from 0.4 s^{-1} in the absence of CDPX to 1.2 s^{-1} with 30 μM CDPX, and remains at this value up to at least 500 μM CDPX. In the presence of CDPX (150 μM) the values of J_A fitted well to a hyperbolic dependence on GABA concentration (equation 2, $n = 1$, where L is the GABA concentration, J_{Amax} is J_A at infinite GABA concentration, and K is the half-response concentration). In the curve fitting procedure, when n was allowed to float with K and J_{Amax} the best fit values were; $n = 0.94 \pm 0.03$, $K = 48 \pm 4$ μM, $J_{max} = 6.8 \pm 0.4$ s^{-1}.

$$\frac{J_A}{J_{Amax}} = (1+(K/L)^n)^{-1} \qquad \text{Eqn. (2)}$$

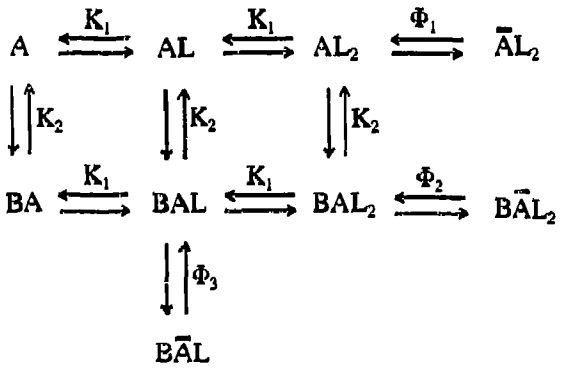
The simplest schemes which fit the data are given in Fig. 3. Values of the constants which predict the results are, for Fig. 3A, $K_1 = 250$ μM, $J_m = 7$ s^{-1}, $\Phi_1 = 0.05$, $\Phi_2 = 0.05$, $\Phi_3 = 0.44$, $K_2 = 10$ μM; or for the scheme in Fig. 3B, $K_1 = 688$ μM, $J_m = 6.8$ s^{-1}, $\Phi_1 = 0.01$, $\Phi_2 = 0.14$, $K_2 = 10$ μM (the lines in Fig. 2A). These numerical values are not unique fits.

4. DISCUSSION

The GABA-mediated initial chloride exchange rate constant, J_A (Fig. 2), which is a measure of the concentration of open channels per unit internal volume (inside the vesicles) [22,23], has been measured for the faster desensitizing receptor in the presence and absence of the benzodiazepine, CDPX. This receptor gives the major fraction (approx. 80%) of the chloride exchange activity in this membrane [14]. The enhancement of chloride exchange due to CDPX (10-fold with 0.3 μM GABA) decreased with increasing GABA concentration (Table I, Fig. 2). In agreement with electrophysiological measurements with different experimental systems [5,10] the maximum rate (at saturation with GABA) was not changed by CDPX. In contrast to other measurements [5,10], the cooperativity was altered. The dependence of chloride exchange rate on GABA concentration became hyperbolic. This indicates that channel opening resulted from the binding of one GABA molecule. For this receptor, the presence of CDPX altered the requirement for optimal channel opening from more than one bound GABA molecule to only one bound GABA molecule. The concentration of CDPX used in these experiments with varying GABA concentration gave the maximal enhancement of chloride exchange rate, which was not decreased by higher CDPX concentrations.

A minimal kinetic scheme previously proposed [15] (Fig. 3A or B, top line) gave a good approximation to $^{36}Cl^-$ exchange rates measured over the whole GABA concentration range in the absence of CDPX. This simplified scheme can be extended to describe the results in the presence of CDPX (Fig. 3). The appropriate scheme must accommodate the following observations: (i) CDPX alone did not mediate chloride exchange in the conditions studied; (ii) CDPX did not inhibit or increase the maximal chloride exchange rate; (iii) CDPX en-

A.



B.

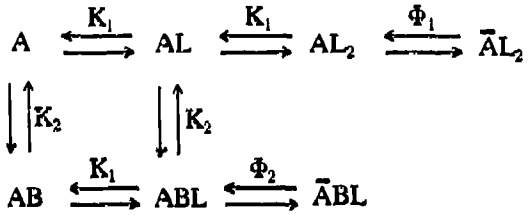


Fig. 3. Minimal kinetic schemes to account for the initial rate of the fast phase (due to the faster desensitizing receptor) of GABA-mediated chloride exchange, J_A. A represents the active (not desensitized) state of the receptor and $\bar{A}$ represents the open channel state of the receptor. K_1 and K_2 are dissociation constants for the binding of GABA (L) and CDPX (B), respectively. Φ_1^{-1}, Φ_2^{-1}, and Φ_3^{-1} are equilibrium constants for channel opening.

(A) The benzodiazepine binds at a site different from a GABA binding site. Channel opening can be mediated by one bound CDPX molecule and one bound GABA molecule or by two bound GABA molecules. The initial chloride exchange rate constant, J_A, is given by the following equation (J_m is the rate constant which would be obtained if all the channels were open)

$$J_A = J_m \cdot f_1/(f_1 + f_2) \qquad \text{Eqn. (3)}$$

where $f_1 = L^2(\Phi_2\Phi_3K_2 + B\Phi_1\Phi_2) + 2LB\Phi_1\Phi_2K_1$ and $f_2 = \Phi_1\Phi_2\Phi_3(K_2+B)(K_1+L)^2$.

(B) This scheme can correspond to two models; (i) CDPX (B) binding causes the protein to convert to a different closed state (AB), which gives channel opening mediated by a single GABA binding site; (ii) CDPX binds at one of the GABA binding sites. Channel opening is mediated by one bound CDPX molecule and one GABA molecule or by two bound GABA molecules. The initial chloride exchange rate constant, J_A, is given by the above equation, where

$f_1 = L^2\Phi_2K_2 + 2LB\Phi_1K_1$

and $f_2 = \Phi_1\Phi_2(K_2 + B)((K_1 + L)^2 - L^2B)$.

hanced the GABA-mediated chloride exchange below saturation; (iv) with CDPX, the chloride exchange approximated a hyperbolic dependence on GABA concentration with this receptor.

A simple hypothesis for the enhancement of GABA-mediated channel opening by CDPX is the binding of CDPX at allosteric effector sites [24–26] to alter the equilibrium of the GABA-mediated (allosteric) transition from the closed to the open channel state. Computed simulations demonstrated that such schemes do not predict a hyperbolic dependence of ion exchange rate on GABA concentration, and in the extreme would give rise to open channel in the absence of GABA [16].

The observed effect of CDPX can be described by a more complicated allosteric mechanism by which CDPX converts the protein complex to another state, closed in the absence of GABA, which forms an open channel on binding one GABA molecule to a single available site. This is represented by the scheme in Fig. 3B, in which **AB** represents the protein with bound CDPX.

Effectively, while two bound molecules are required for channel opening, one of these is CDPX. We should note that there is a conceptually different hypothesis, which is also represented by Fig. 3B and cannot be excluded by this data alone. This involves the binding of CDPX to one of the channel opening binding sites to give channel opening when GABA is bound to the other GABA binding site. If this were the case, CDPX must bind to only one of the GABA binding sites, since inhibition at high CDPX concentration was not observed with this receptor. In an elaboration of this model, CDPX could bind to a site other than the GABA site and channel opening could be mediated by two bound ligands, one of which could be CDPX. This is represented in Fig. 3A and predicts approximately hyperbolic response at low GABA concentrations. These models of CDPX acting as a channel-opening ligand do not involve the postulation of allosteric transformations other than channel opening, unlike the first model presented.

Preliminary data indicate that the other receptor present on these membrane vesicles, which gives slower chloride exchange and undergoes slower desensitization [14,15], is also enhanced in chloride exchange activity by CDPX. But hyperbolic behavior is not observed and the invoivement of at least two bound GABA molecules in channel opening is retained in the presence of CDPX. Thus the molecular mechanism of the enhancement of channel opening by benzodiazepines may differ with different versions of $GABA_A$ receptors.

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