Desensitization of a \( \gamma \)-Aminobutyric Acid Type A Receptor in Rat Is Increased by Chronic Treatment with Chlordiazepoxide: A Molecular Mechanism of Dependence\(^1\)

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ABSTRACT

When rats were made tolerant to the benzodiazepine tranquilizer chlordiazepoxide (CDPX) by its steady administration, a particular \( \gamma \)-aminobutyric acid type A (GABA\(_A\)) receptor in cerebral cortex was modified. Its rate of desensitization in the absence of CDPX was enhanced (3-fold with 10 \( \mu \)M GABA) below saturation with GABA, and the dependence of this rate on GABA concentration was changed from sigmoid to hyperbolic. This mimicked the effect of the presence of CDPX on desensitization of the naive receptor. This receptor has been characterized by its rapid desensitization (\( t_{1/2} = 30 \text{ msec at saturation} \)). In contrast, a different, slower desensitizing GABA\(_A\) receptor, on the same membrane, was unaffected, and the initial transmembrane halide exchange rate of the faster desensitizing receptor was unaltered. In the presence of CDPX, the initial halide exchange rate of the modified receptor was enhanced; but the already enhanced desensitization rate was not altered. During chronic presence of CDPX and the development of tolerance, the total signal due to this receptor remained constant at the value before exposure. After discontinuation, the total signal decreased but could be restored to the original value by the presence of CDPX. It was postulated that dependence and withdrawal syndromes result from a decreased ratio of initial chloride flux rate to desensitization rate, caused by an increase in desensitization. The contribution of this effect in vivo would depend on desensitization making a contribution to signal termination (or the fraction of receptors that are inactive (desensitized)).

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ABBREVIATIONS: GABA, \( \gamma \)-aminobutyric acid; CDPX, chlordiazepoxide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
Chronic administration of these drugs causes neurochemical as well as behavioral changes (Gallager and Primus, 1993; Gallager and Tallman, 1990; Miller, 1991; Rosenberg and Chiu, 1985). In particular, in rats and mice, continuous presence of these drugs produces changes in ligand binding as well as channel opening properties of GABA receptor (Gallager et al., 1985; Hernandez et al., 1990; Tietz et al., 1993). The magnitude of these effects increases with the pharmacological efficacy of the benzodiazepine.

These observations were complex. (a) Measurements of various biochemical and functional properties of the receptor changed with different time courses. (b) The changes in receptor properties depended on the protocol of the chronic administration (e.g., continuous or intermittent, injected or inserted). (c) The changes varied in different brain regions. (d) They varied with the individual drug chronically administered. (e) They varied with the testing protocol and with the ligand used to assay the effects on the receptor. Evidently, tolerance and dependence are complex responses including series of different events at different types of receptor. Observations of changes in rates of protein subunit synthesis suggested that a change in subunit composition might occur but at a rate too slow to account for the early changes of observations of changes in rates of protein subunit synthesis. (e.g., continuous or intermittent, injected or inserted). (c) The changes varied in different brain regions. (d) They varied with the individual drug chronically administered. (e) They varied with the testing protocol and with the ligand used to assay the effects on the receptor. Evidently, tolerance and dependence are complex responses including series of different events at different types of receptor. Observations of changes in rates of protein subunit synthesis suggested that a change in subunit composition might occur but at a rate too slow to account for the early changes of function observed (Heninger et al., 1990; Kang et al., 1994; Kang and Miller, 1991; Primus and Gallager, 1992). The rates of the initial changes caused by chronic administration and also by discontinuation suggested that modification of the receptor in the membrane occurs.

A measure of GABA<sub>A</sub> receptor function has been the GABA-mediated uptake of $^{36}$Cl<sup>−</sup> into sealed vesicles formed from membrane of disrupted cells (Allan et al., 1985; Harris and Allan, 1985; Subbarao and Cash, 1985). In these measurements, radiotracer ion transport is due to specific anion exchange through the receptor channel and is a function of two different responses, channel opening and desensitization of the receptor. Using a rapid chemical kinetics technique, quench flow, the initial halide-exchange rate through open channel of GABA<sub>A</sub> receptor and the rate of its desensitization have been resolved (Cash and Subbarao, 1987b, 1987c). Quench-flow ion flux methods are suitable for investigating changes in a receptor due to drug administration, learning or disease because the membrane suspension can be made directly from brain and can be mixed rapidly with solutions of known and controlled concentrations.

We are investigating changes accompanying tolerance to CDPX in a native membrane preparation from rat cerebral cortex, in which two GABA<sub>A</sub> receptors have been distinguished by their desensitization rates (Cash and Subbarao, 1987a, 1987c). After the addition of GABA, these receptors mediate transmembrane $^{36}$Cl<sup>−</sup> exchange, which proceeds in two phases, each terminated by a desensitization process. This is described by equations 1 to 3, in which $J_A$ and $J_B$ are the initial rate constants for ion exchange, a measure of open channel concentration, and $\alpha$ and $\beta$ are rate constants for desensitization of the faster desensitizing and slower desensitizing receptors, respectively: $[^{36}X]_b/t[^{36}X]_a$ is the fractional transmembrane equilibration of isotopic specific activity at time $t$ (e.g., see figs. 1–3). The faster desensitizing receptor exhibits higher initial activity (≈80% of total channel opening activity; $J_A/J_B \sim 5$), such that the initial signal intensity is desensitized to reveal a slower desensitizing signal due to the second receptor (equation 4). These two phases of halide exchange are sufficiently separated in time for the four rate constants (equations 2b and 3b) to be determined. The ion-exchange rate constant $J_A$ (equation 4) is a measure of the number of open channels at time $t$ and initially has the value $J_A + J_B$. The functions $A$ and $B$ (equations 2b and 3b) pertaining to the two receptors, respectively, are given directly by the quench flow measurements (equation 1) and are equal to the area under the curve of $J_A$ plotted against time, shown in fig. 6 for the single receptor type, A. The value of $A$ represents the open channel integrated over time and is a dimensionless factor that determines the size of the signal up to time $t$ and the total signal. Total signal is relevant where a number of signals contribute to a summed membrane potential.

\[
[^{36}X]_b = 1 - e^{-(A+B)t}
\]  

\[
A = \int_0^t e^{-xt}dt = J_A\left(1 - e^{-xt}/\alpha\right)
\]  

\[
lim A = \frac{J_A}{\alpha}
\]  

\[
B = \int_0^t e^{-\beta t}dt = J_B\left(1 - e^{-\beta t}/\beta\right)
\]  

\[
lim B = \frac{J_B}{\beta}
\]  

\[
J_A = J_Ae^{-xt} + J_Be^{-\beta t}
\]  

In studies with this membrane preparation, CDPX gave an enhancement of halide exchange (Serfozo and Cash, 1992) as
well as desensitization (Cash and Serfozö, 1995) rates of both GABA_A receptors at less than saturating GABA concentrations. Furthermore, CDPX changed the dependence on GABA concentration of both J_A and J_α from a sigmoid (cooperative) shape to an approximately hyperbolic (noncooperative) dependence. This extended the response curves to lower concentrations, so the factor by which J_A and J_α were increased by CDPX became larger with decreasing GABA concentration. Here, we report that chronic administration of CDPX, making the rat tolerant to benzodiazepines, leads to an increase (3-fold with 10 μM GABA) of desensitization rate, J_α of the faster desensitizing receptor with a change in its dependence on GABA concentration from sigmoid to hyperbolic, in the absence of exogenous CDPX, in tolerant rat.

Methods

Drug administration. Sprague-Dawley male rats (6–8 weeks old) were implanted subcutaneously with osmotic minipumps (Alzet; Alza, Palo Alto, CA) calibrated to deliver CDPX (10 mg/kg/day) (Sigma Chemical, St. Louis, MO).4 Periods of chronic treatment of 7 or 14 days gave the same results. Control rats, whose implants contained delivery vehicle alone, gave the same results as naive rats. The concentration of CDPX in the blood plasma, determined5 at the time of decapitation, was 0.120 ± 0.001 μg/ml. This regimen produced measurable tolerance as demonstrated by a 2.4-fold increase in CDPX anxiolytic dose using an elevated-plus-maze test evaluated in a separate group of rats (data not shown). This tolerance to anxiolytic effect was determined soon after the pump had expired and before the development of any signs of withdrawal. Initial anxiolytic dose was determined with naive animals. However, this pharmacological “dose equivalency” was 5- to 13-fold lower than in other recent studies with different drugs (Allan et al., 1992; Hu and Ticku, 1994b; Li et al., 1993; Yu et al., 1988). We previously reported that measurements of GABA-mediated 36Cl flow do not depend on the membrane preparation method with naive rat (Cash and Subbarao, 1989). We have now shown that the results are the same with our preparation and the Microsac preparations with tolerant as well as naive rat. In addition, they are similar over a range of membrane protein concentrations (âµg/ml)

Radioisotope uptake experiments. The membrane suspension and all solutions were in solution B. The incubations were made in a quench flow machine with an in-line decelerating filtration setup (Cash et al., 1991; Cash and Hess, 1981). The membrane was kept at 0° and warmed to 30° in 2 min after being loaded and was allowed to stand for an additional 1 min before actuation. Channel opening was initiated by mixing the membrane (protein concentration, 750 μg/ml) with an equal volume (225 μl) of solution containing GABA and radiotracer. Influx of 82Br (12.5 μCi/ml) (Missouri University Research Reactor, Columbia, MO) (Cash et al., 1995; Cash and Serfozö, 1995) or 36Cl (7.5 μCi/ml) (New England Research Products, Boston, MA) (Allan et al., 1985; HARRIS and Allan, 1985; Subbarao and Cash, 1985) was measured. After a predetermined incubation time, the specific ion influx was terminated at the time indicated (on the abscissa) by mixing with 225 μl of bicuculline N-methiodide (3 mM) (Cash and Subbarao, 1987b), an inhibitor of channel opening. The mixture was passed immediately through a glass-fiber filter disk (No. 31, Schleicher & Schuell, Keene, NH).7 The membrane, which was completely retained on the disk, was washed with solution B (3 × 10 ml) and dried, and the internalized radioactivity was counted with scintillation fluid. In the case of 82Br, the counts were corrected for 82Br decay (t_1/2 = 35.3 hr) by normalizing to the first count (minus the counter background) using the equation: cpm (corrected) = (cpm – counter background)/exp(-λt/2) time elapsed (hr)/

6 Mice were made tolerant by chronic treatment with lorazepam (6.8 mg/kg/day) for 7 days. The rate of desensitization of the faster desensitizing receptor (α) in tolerant mice, in the absence of CDPX, had been increased 3.4-fold (relative to naive mice) from α = 0.8 to 2.7 sec^−1 after the chronic treatment, whereas the desensitization rate of the slower desensitizing receptor (β) and the halide exchange rates (J_α and J_β) of both receptors were unaltered. In the presence of CDPX, the already enhanced rate of desensitization (α) of tolerant mice was not increased by CDPX, whereas β, J_α and J_β were increased in the same way as in naive mice. In naive mice, α was increased in the presence of CDPX (150 μM) by 1.9-fold to 1.5 sec^−1. In both tolerant and naive mice, the rate constants were, in the absence of CDPX, J_α = 0.54 sec^−1, J_α = 0.65 sec^−1 and β = 0.03 sec^−1; in the presence of CDPX (150 μM), the rate constants were J_α = 1.35 sec^−1, J_β = 0.09 sec^−1 and β = 0.17 sec^−1.

7 Disks supplied by other manufacturers since 1989 were not satisfactory due to quenching and precise results. Disks that had performed satisfactorily in our previous work resembled (in appearance and performance) those currently available from Schleicher & Schuell rather than Fisher or Whatman.

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4 The osmotic pumps were inserted (by A.M.A.) at the Department of Psychology, Washington University, St. Louis, and the implanted rats were transported to the University of Missouri.

5 The concentration of CDPX in the blood was steady throughout the chronic treatment. The active metabolites, after 14 days, were demethylchloralosezoxide (13.6 μg/ml) and demeclozipam (0.008 μg/ml). CDPX and its major metabolites were determined by high-performance liquid chromatography (Laster et al., 1983).

6 Mice were made tolerant by chronic treatment with lorazepam (6.8 mg/kg/day) for 7 days. The rate of desensitization of the faster desensitizing receptor (α) in tolerant mice, in the absence of CDPX, had been increased 3.4-fold (relative to naive mice) from α = 0.8 to 2.7 sec^−1 after the chronic treatment, whereas the desensitization rate of the slower desensitizing receptor (β) and the halide exchange rates (J_α and J_β) of both receptors were unaltered. In the presence of CDPX, the already enhanced rate of desensitization (α) of tolerant mice was not increased by CDPX, whereas β, J_α and J_β were increased in the same way as in naive mice. In naive mice, α was increased in the presence of CDPX (150 μM) by 1.9-fold to 1.5 sec^−1. In both tolerant and naive mice, the rate constants were, in the absence of CDPX, J_α = 0.54 sec^−1, J_α = 0.65 sec^−1 and β = 0.03 sec^−1; in the presence of CDPX (150 μM), the rate constants were J_α = 1.35 sec^−1, J_β = 0.09 sec^−1 and β = 0.17 sec^−1.

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35.3)). Unspecific uptake, measured in the same way in the absence of GABA, was subtracted from total uptake to give the GABA-mediated specific influx. Each data point shown gives the difference between mean values of triplicate determinations made in the presence and absence of GABA.

The precision of the GABA-mediated uptake is given by

\[ \frac{\sigma}{\sigma_{\text{total}}} = 0.05 \] (16) Using \( {\Delta}^{3} \text{Br}^{-} \), the precision of the GABA-mediated uptake was \pm 4% (7% for \( \text{^{38}Cl}^{-} \)), and that of the total uptake was 2.7% (5% for \( \text{^{38}Cl}^{-} \)), with a total count of typically 2750 to 13,000 counts/10 min (2000–3000 for \( \text{^{38}Cl}^{-} \)). The maximum signal-to-background ratio was \pm 2.0. The GABA-mediated uptake was expressed as a percentage of the equilibrium count, typically 6000 counts/10 min (1800 for \( \text{^{38}Cl}^{-} \)). The lower precision with \( \text{^{38}Cl}^{-} \) was due to the lower specific activity generally used. Quenching with N-methyl bicuculline was shown to be sufficiently rapid (Cash and Subbarao, 1987c). The radioisotope \( {\Delta}^{3} \text{Br}^{-} \) has been shown to have the same GABA-mediated permeability as \( \text{^{38}Cl}^{-} \) in these experiments (Cash et al., 1995). It has the advantages of economy as well as a short \( t_{1/2} \) of 35 hours, alleviating contamination and disposal problems.

Enhancements of channel opening and desensitization by CDPX were studied primarily with 10 \( \mu \text{M} \) GABA (figs. 1 and 2), a concentration at the foot of the response curves (~5% of the maximum halide-exchange and desensitization rates), where the enhancement was large (Cash and Serfözö, 1995; Serfözö and Cash, 1992) and both \( J_{A} \) and \( \alpha \) can be determined from the same isotope uptake progress curve. Measurements covered the entire time scale of the response, so the rate constants characterizing the second phase of halide exchange, \( J_{B} \) and \( \beta \), could be determined and separated from \( J_{A} \) and \( \alpha \). The values in naive rat were in good agreement with those determined by the preincubation method (Cash and Serfözö, 1995); this supports the validity of the assumptions of homogeneity underlying the kinetic analysis with these membrane vesicles (Cash et al., 1988).

**Fig. 1.** Effect of CDPX on GABA-mediated transmembrane halide exchange in cerebral cortical membrane from naive rat. The progress of influx of radiotracer, into native membrane vesicles, after the addition of GABA (10 \( \mu \text{M} \), at 30° was measured in the absence (open symbols) and presence (closed symbols) of CDPX (150 \( \mu \text{M} \)). The presence of CDPX caused a ~2.5-fold increase in the rates of both halide exchange and desensitization for both the receptors, but at times of >3 sec, this did not cause a significant difference in the measurement because desensitization rate as well as initial ion exchange rate was increased. Four quench-flow experiments (see Methods) with preparations from different animals are indicated by the different symbols. Influx of \( {\Delta}^{3} \text{Br}^{-} \) (12.5 \( \mu \text{Ci/ml} \)) was measured, except in one experiment (\( \Delta \), A) where \( \text{^{38}Cl}^{-} \) (7.5 \( \mu \text{Ci/ml} \)) was used. Fitted lines were computed from equations 1, 2b and 3b with the values of the rate constants given in table 1. Radiotracer influx is expressed as a percentage of the equilibrium count. Protein concentration was 375 \( \mu \text{g/ml} \).

**Desensitization and Drug Dependence**

In naive rat, the initial open channel concentration of the faster desensitizing receptor (\( J_{A} \) value) was increased with increasing GABA concentration. However, the rate of desensitization was also increased, so the total open channel (total signal,\( ^{3} \text{A} \), equation 2, see fig. 6) remained practically unchanged, although the response was shifted to shorter times. Thus, desensitization contrived to keep the total signal independent of GABA concentration. Total signal depended on the number of GABA receptors involved, whereas initial signal intensity\( ^{3} \) increased with increasing GABA concentration as well as the number of receptors.

In the presence of CDPX (150 \( \mu \text{M} \)), the rates of both GABA receptor-mediated initial halide exchange, \( J_{A} \), and desensitization, \( \alpha \), were increased ~3-fold with 10 \( \mu \text{M} \) GABA (fig. 1; table 1). Initial signal intensity was increased and the signal was shifted to shorter times by the increased desensitization rate (see fig. 6), so the total signal of the faster desensitizing receptor (\( t_{1/2} = 33 \text{ msec at saturation} \) was practically unchanged by CDPX. This effect was equivalent to an increase in GABA concentration from 10 to ~30 \( \mu \text{M} \). In the presence of CDPX, as in its absence, the total signal was independent of GABA concentration.

The progress of halide exchange, in the presence of added CDPX (150 \( \mu \text{M} \)) with 10 \( \mu \text{M} \) GABA, was similar for naive and tolerant rat (figs. 1 and 2, filled symbols), corresponding to practically no change in initial or total signal when the rat became tolerant. Total signal remained virtually independent of GABA concentration for this receptor.

With tolerant rat in the absence of CDPX, the initial halide exchange rate, \( J_{A} \) (and the specific rates,\( ^{3} \) \( J_{B} \) and \( \beta \) of the
slower desensitizing receptor \( (t_{\text{d}} = 530 \text{ msec at saturation}) \) reverted to the same values and followed the same sigmoid dependence on GABA concentration as with naive rat, but the rate constant for desensitization of the faster desensitizing receptor, \( \alpha \), remained enhanced in the absence of CDXP, with a value near 2.0 sec\(^{-1}\) (10 \( \mu \text{M GABA} \)). This is marginally larger than the value observed in the presence of CDXP in naive rat and 2.9-fold larger than the value in naive rat in the absence of CDXP (table 1). In tolerant rat, the dependence of \( \alpha \) on GABA concentration (figs. 3 and 4) was approximately hyperbolic, although there was no CDXP in the reaction solutions. This behavior was the same as with membrane from naive or tolerant rats in the presence of the drug. In other words, in the tolerant rat, the enhancement of \( \alpha \) by CDXP in the naive rat was replicated in the absence of the drug (table 1). The analyses, showing absence of a significant quantity of CDXP and its active metabolites in the membrane preparation, were supported by the normal, unaffected values of \( J_A, J_B\) and \( \beta \), which are known to be increased in the presence of CDXP.

In the absence of CDXP, the total signal of the tolerant, faster desensitizing receptor was reduced to less than that of naive rat (2.9-fold decrease with 10 \( \mu \text{M GABA}\)) because of its enhanced desensitization rate. Total signal increased with increasing GABA concentration because desensitization rate followed a hyperbolic dependence on GABA concentration, whereas ion flux rate followed a steeper, sigmoid dependence (fig. 4). This differs from naive receptor in the absence or presence of CDXP, and from tolerant receptor in the presence

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**TABLE 1**

**Effect of CDXP on the rates of halide exchange and receptor desensitization**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Naive ( ^a )</th>
<th>Tolerant ( ^a )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Faster desensitizing receptor ( t_{\text{d}} = 30 \text{ msec at saturation with GABA} )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ion exchange rate, ( J_A^b )</td>
<td>No CDXP</td>
<td>0.45 ( \mu \text{sec}^{-1} )</td>
</tr>
<tr>
<td>150 ( \mu \text{M CDXP} )</td>
<td>1.07 ( \mu \text{sec}^{-1} )</td>
<td>1.06 ( \mu \text{sec}^{-1} )</td>
</tr>
<tr>
<td>Desensitization rate, ( \alpha^c )</td>
<td>No CDXP</td>
<td>0.68 ( \mu \text{sec}^{-1} )</td>
</tr>
<tr>
<td>150 ( \mu \text{M CDXP} )</td>
<td>1.8 ( \mu \text{sec}^{-1} )</td>
<td>2.0 ( \mu \text{sec}^{-1} )</td>
</tr>
<tr>
<td>Slower desensitizing receptor ( t_{\text{d}} = 530 \text{ msec at saturation with GABA} )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ion exchange rate, ( J_B^b )</td>
<td>No CDXP</td>
<td>0.05 ( \mu \text{sec}^{-1} )</td>
</tr>
<tr>
<td>150 ( \mu \text{M CDXP} )</td>
<td>0.13 ( \mu \text{sec}^{-1} )</td>
<td>0.12 ( \mu \text{sec}^{-1} )</td>
</tr>
<tr>
<td>Desensitization rate, ( \beta^d )</td>
<td>No CDXP</td>
<td>0.05 ( \mu \text{sec}^{-1} )</td>
</tr>
<tr>
<td>150 ( \mu \text{M CDXP} )</td>
<td>0.17 ( \mu \text{sec}^{-1} )</td>
<td>0.17 ( \mu \text{sec}^{-1} )</td>
</tr>
</tbody>
</table>

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a With 10 \( \mu \text{M GABA} \) at 30°C.

b GABA (before desensitization) values of first-order rate constants \( k_a \) for transmembrane exchange (uptake) of radiolabeled halide.

c First-order rate constants \( k_a \) for desensitization (decrease in \( J_A \), from values of \( J_A \) or \( J_B \)).

d Initial (before desensitization) values of first-order rate constants \( k_a \) for transmembrane exchange (uptake) of radiolabeled halide.

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### References

1. **Fig. 3.** Dependence on GABA concentration of halide exchange in cerebral cortical membrane from tolerant rat (closed symbols, continuous lines) vs. naive rat (open symbols, dotted lines) in the absence of CDXP. Influx of \( ^{36}\text{Br}^- \) (12.5 \( \mu \text{Ci/ml} \)) into the membrane vesicles was measured. With 10 \( \mu \text{M GABA} \), the faster phase of halide exchange was desensitized faster in tolerant rat (●) than naive rat (○). With 40 \( \mu \text{M GABA} \), the progress of radioisotope exchange in tolerant rat (●) and naive rat (○) were not distinguishable, and with 1000 \( \mu \text{M GABA} \), the halide exchange progressed only marginally more slowly in tolerant rat (▲) than naive rat (△). The tolerant rats had been administered CDXP for 15 days with an implanted osmotic pump. The fitted lines were computed from equations 1, 2b and 3b with the following rate constant values: 10 \( \mu \text{M GABA} \), for tolerant rat \( \alpha = 1.5 \text{ sec}^{-1} \), for naive rat \( \alpha = 0.6 \text{ sec}^{-1} \), with both tolerant and naive, \( J_A = 0.5 \text{ sec}^{-1} \), \( J_B = 0.05 \text{ sec}^{-1} \) and \( \beta = 0.03 \text{ sec}^{-1} \). With 40 \( \mu \text{M GABA} \), for both tolerant and naive, \( J_A = 1.4 \text{ sec}^{-1} \), \( \alpha = 4.5 \text{ sec}^{-1} \), \( J_B = 0.4 \text{ sec}^{-1} \) and \( \beta = 0.3 \text{ sec}^{-1} \). With 1000 \( \mu \text{M GABA} \), for tolerant rat \( \alpha = 15 \text{ sec}^{-1} \); for naive rat \( \alpha = 17 \text{ sec}^{-1} \), with \( J_A = 8.5 \text{ sec}^{-1} \), \( J_B = 2.2 \text{ sec}^{-1} \) and \( \beta = 1.2 \text{ sec}^{-1} \). Radiotracer influx is expressed as a percentage of the equilibrium count, 5300 counts/10 min. Protein concentration was 300 \( \mu \text{g/ml} \).

### Notes

- **Fig. 4.** Dependence of desensitization rate on GABA concentration in tolerant and naive rats: plot of desensitization rate constant \( \alpha \) per GABA concentration \( [\text{GABA}] \) against desensitization rate constant (analogous to Eadie-Hofstee plot for enzyme reaction rates). The lines radiating from the origin are isomolar in [GABA] labeled in \( \mu \text{M} \). The rate constant for desensitization \( \alpha \) was obtained from the progress of radiotracer influx by fitting equations 1, 2b and 3b to curves exemplified in figures 1 to 3. A, In the absence of CDXP, with membrane from naive rat (○) untransformed determinations of \( \alpha \) at low [GABA], relative to naive rat (●), In the presence of CDPX, to tolerant and naive receptors, gave similar variations of \( \alpha \) on GABA concentration. B, In the presence of CDXP (150 \( \mu \text{M} \)) with naive rat (●), the dependence of \( \alpha \) on GABA concentration was approximately hyperbolic (linear on this plot), giving enhanced desensitization rates at less than saturating [GABA]. C, With membrane from tolerant rat in the absence of CDXP (△), an approximately hyperbolic dependence of \( \alpha \) on GABA concentration remained, with an enhanced value of \( \alpha \) at low [GABA], relative to naive rat (○). In the presence of CDXP, tolerant and naive receptors, gave similar variations of \( \alpha \) on GABA concentration (line B). The lines were computed from the equation derived from the minimal model (fig. 5, legend) and were fitted to untransformed determinations of \( \alpha \). The effect of CDXP (□ to △) could be described by a change in value of only one of the constants in the model; a decrease in \( K_i \) to \( K_{i(CDXP)} \) (or an increase in \( k_i \) to \( k_{i(CDXP)} \)). The measurements are fitted with values of the constants, defined in figure 5: for naive rat: \( K_i = 70 \mu \text{M}; K_{i(CDXP)} \leq 5 \mu \text{M}; K_0 = 50 \mu \text{M}; k_0 = 21 \text{ sec}^{-1} \); \( K_i \), relatively very small. If \( K_i \) and \( K_0 \) are independently variable, \( k_i \) may be relatively very small.
Fig. 5. Minimal kinetic model that describes the dependence of desensitization rate on GABA concentration, in naive and tolerant rats, in the presence and absence of CDPX (Cash and Serfözö, 1995). The active state (A) binds two molecules of GABA (L) (with dissociation constants $K_1$ and $K_2$, respectively) to give a doubly ligated species largely in the open channel state ($\text{AL}_2$). Much more slowly than ligand binding or channel opening, the ligated species undergo conversion (with rate constants $k_1$ and $k_2$) to desensitized receptor (DL and DL$\text{L}_2$), which has negligible channel opening activity. The brackets in the reaction scheme denote that $K_1$ and $K_2$ are coefficients of $[\text{AL}_2 + \text{AL}]$.

The change from sigmoid to hyperbolic dependence on [GABA], due to binding CDPX, can be described by a single change in the singly ligated receptor (fig. 4, legend). The rate constant for desensitization is given by:

$$\frac{d[\text{AL}_2]}{dt} = -k_1[\text{AL}] + k_2[\text{DL}_2].$$

This simplified minimal kinetic model is used to describe the present observations and is not intended to be a complete description of the mechanisms of the receptor.

Discussion

The faster desensitizing receptor ($t_\text{s} = 30$ msec at saturation) contributes the major portion (80%) of the GABA$_A$ receptor activity in the membrane studied (Cash and Subbarao, 1987b, 1987c). In rats made tolerant to CDPX, there was an increase (3-fold with 10 $\mu$M GABA) in desensitization rate, $\alpha$, of this receptor, which persisted after the removal of CDPX when the other responses of GABA$_A$ receptor had returned to normal. The structural change of this GABA$_A$ receptor in tolerant animal may be related to that which causes an acute enhancement of desensitization in naive rat in the presence of CDPX. An explanation is that chronic exposure to CDPX produces a change in the receptor, which persists after the removal of the drug and prevents the immediate reversion to the normal state ($\alpha$ value) seen with naive receptor in the absence of drug. During the chronic treatment, change or changes must have occurred that prevented or retarded the conformational relaxation (which is relatively very rapid in naive animals) from the tolerant state after removal of the drug. These changes might involve phosphorylation of phosphorylation site(s) on the polypeptide, which may influence the rates of particular conformational changes or the relative stabilities of particular conformations.

The rates of halide exchange and desensitization over the entire GABA concentration range of response could be described by a minimal kinetic model shown in fig. 5 (Cash and Subbarao, 1987b, 1987c; Cash and Serfözö, 1995). There are two possibilities for the subsaturation rate enhancements, both of which involve a change in the receptor with one GABA molecule bound but not with two GABA molecules bound to the pertinent sites. The receptor is modified by the binding of CDPX to either (1) increase the affinity of the receptor for the first GABA molecule bound or (2) increase the rates of desensitization and halide exchange with only the first GABA molecule bound. Both these possibilities could cause the response to be determined predominantly by a single GABA molecule binding to a single site (Cash and Serfözö, 1995; Serfözö and Cash, 1992), giving rise to an approximately hyperbolic dependence of rate on GABA concentration. Explanation 1 might be favored because of its simplicity and also, at least for channel opening, because of some recently reported electrophysiological measurements.
studies were of a different GABAA receptor in a different experimental system, with low GABA concentration. Because there can be significant differences between various subtypes of GABAA receptor, this argument can only be tentative.

The presence of GABA normally causes an increase in binding of benzodiazepine by GABAA receptor; this has been called "the GABA shift." This effect was attenuated after chronic treatment with benzodiazepine drugs (Hu and Ticku, 1994a; Little et al., 1987; Tietz et al., 1989). This attenuation has been called "allosteric uncoupling of GABA binding sites from benzodiazepine sites." Those observations are consistent with the present measurements, which indicate that a conformational state, which corresponds to an increased GABA affinity, is already present in the tolerant receptor in the absence of CDPX.

The presence of benzodiazepines generally causes an increase in GABA-mediated 36Cl– uptake into a membrane suspension. There is consensus in published reports that this effect is attenuated after chronic administration of these drugs leading to tolerance (Allan et al., 1992; Hu and Ticku, 1994b; Li et al., 1993; Yu et al., 1988). This has been called "allosteric uncoupling of benzodiazepine binding sites from channel opening." It was not observed in the present measurements (figs. 1 and 2). However, the condition required for that behavior would be met if Jw were held in its enhanced state, like a. This also would explain why the decreased GABA-mediated 36Cl– uptake by tolerant receptor, observed here in the absence of drug, was small or nonexistent in those studies. The reason why only a was elevated in our experiments might be our use of a less potent drug in a concentration 5 to 13 times below its dose equivalence to those in the cited reports. In any case, our observations of behavioral tolerance and receptor function show that loss of benzodiazepine enhancement ("uncoupling") of the 36Cl– exchange, seen in the assay of several seconds, is not a requirement for behavioral tolerance. The decrease of 36Cl– exchange was due to increased desensitization, with no change in the channel opening process or receptor density.

While there was no change in channel opening, the specific enhancement of desensitization, with a change in its dependence on GABA concentration, demonstrated separate control of desensitization and channel opening. These two different responses, mediated by the same neurotransmitters and inhibited or enhanced with similar pharmacology, normally occur together, although with an estimated 50-fold difference in rate. If the explanation above is correct (fig. 5; decrease in K1; hypothesis 1), then channel opening and desensitization must be mediated by different GABA binding sites because desensitization was affected independently from channel opening. The two responses must be mediated by at least different structural domains and possibly different subunits. On the other hand, if the mechanism of enhancement of desensitization were an increase of the desensitization rate of AL (fig. 5; increase in k2; hypothesis 2), then a rate-limiting step on the route to desensitization but not to channel opening must have been accelerated in tolerant rat.

In summary, this tolerant receptor is functionally different from naive receptor in the following ways. First, the signal covers a shorter time range due to faster desensitization. Second, the total signal is smaller for the same reason. Third, CDPX increases the total signal, as well as initial signal intensity, because it increases channel opening but not desensitization rate. Fourth, CDPX does not decrease the time scale of the signal for the same reason. Fifth, increasing GABA concentration increases the total signal, in the absence of CDPX, because ion flux rate is increased more steeply than desensitization rate (sigmoid dependence vs. hyperbolic dependence on [GABA]).

To what extent these chronic effects of CDPX might contribute to the response of this receptor in vivo would depend on the contribution of desensitization to limitation of the signal; this would depend on the relative rates of GABA removal and desensitization. First, the change in the receptor would have an effect, only where the difference between naive and tolerant receptor is significant, below 100 μM GABA (fig. 4). Second, a role played by desensitization in signal termination or in controlling the fraction of receptor that is active (capable of forming an open channel) would increase with increased exposure to GABA. This would increase with decreasing rate of GABA removal, which occurs by diffusion and uptake by Na-GABA symport. Removal of GABA would depend on the synaptic morphology because this provides the limitations to diffusion of neurotransmitter from the synapse. Moreover, the importance of desensitization would be increased by decreased activity of the neurotransmitter uptake mechanism because this would affect the concentration gradients of diffusing neurotransmitter. Factors decreasing neurotransmitter uptake rate would enhance the postulated roles of desensitization, the independence of total signal on neurotransmitter or drug concentration and the chronic effect of the drug.) Third, desensitization would have an increased contribution to signal termination where presynaptic GABA release is greater because desensitization rate would be faster (fig. 4) and there would be more GABA to be removed.

Computer simulations indicated that even when the signal is cut short by a factor other than desensitization (decrease of [GABA] at the receptor) but some contribution from desensitization remains, tolerant receptor would still give a smaller total signal than naive receptor. And the presence of CDPX would increase the signal. Where desensitization makes a significant contribution to signal termination, the initial effects of the drug on naive receptor would be to increase the initial signal intensity, without similarly increasing the total signal. While the drug remains present, there would be practically no further change in receptor response, at least in the time range of chronic treatment studied here. On withdrawal of the drug, the total signal of the tolerant receptor would be decreased below that of naive receptor, and its initial intensity would be decreased to the value of naive receptor. To obtain a normal (as in naive) total signal in the tolerant animal, presence of the drug would be required.

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