Modulation of GABA-gated Chloride Ion Flux in Rat Brain by Acute and Chronic Benzodiazepine Administration

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ABSTRACT
γ-Aminobutyric acid (GABA)-gated Cl⁻ influx was studied in rat brain "microsacs." Midazolam caused a shift to the left of the GABA log dose-response curve. Pentobarbital produced a similar shift plus an increase in maximum response. Diazepam, flurazepam and desalkyflurazepam also enhanced GABA-gated Cl⁻ flux. Their effects were blocked by Ro15-1788, a benzodiazepine antagonist. Acute GABA pretreatment caused a shift to the left of the GABA dose-response curve but had no effect on the ability of benzodiazepines or pentobarbital to increase GABA-gated Cl⁻ influx. In rats made tolerant by 4 weeks of flurazepam treatment, there was no decrease in the ability of GABA to modulate Cl⁻ flux. GABA was more potent in microsacs from nonwithdrawn rats. In rats withdrawn for 12 but not 48 hr, the maximum GABA response was increased. The ability of benzodiazepines and of pentobarbital to enhance GABA-gated Cl⁻ influx was reduced, showing tolerance. However, 2 days after withdrawal from chronic treatment, this was no longer statistically significant. The results show that benzodiazepine tolerance involves reduced functional coupling between the benzodiazepine recognition site and the GABA recognition site-CI⁻ channel. Furthermore, reduced effectiveness of GABA, agonists in benzodiazepine-tolerant animals might result from alterations in neuronal activity that occur subsequently to activation of the GABA receptor-gated anion channel.

The effects of anxiolytic BZs are mediated by specific binding sites on neurons (Möhler and Okada, 1977a,b; Squires and Braestrup, 1977). Binding of BZs to these sites was found to be modulated by GABA (Tallman et al., 1978; Chiu and Rosenberg, 1979; Wong and Iversen, 1985). Conversely, GABA binding (lower affinity state of its receptor) was modulated by the binding of BZs to their recognition sites (Skerritt et al., 1982; Paldi-Haris et al., 1985; Corda et al., 1986). These results suggested that BZ and GABA recognition sites may be linked allosterically in a single macromolecule. Indeed, purified receptors have been shown to contain both GABA and BZ recognition sites (Sigel and Barnard, 1984).

Anion channels coupled to GABA_A receptors open in bursts when activated by GABA or GABA mimetics (Choi et al., 1981; Hamil et al., 1983), which results in an influx of chloride ions (Krnjevic, 1974). This can be measured by 36Cl⁻ influx into brain "microsacs" (Allan and Harria, 1986a,b; Yu et al., 1986; Obata and Yamamura, 1986) and cultured chick neurons (Tehrani et al., 1986; Ticku et al., 1986) or efflux of 36Cl⁻ from preloaded rat hippocampal slices (Wong et al., 1984) and synaptoneurosomes (Schwartz et al., 1985). Because the actions of BZs and barbiturates are expressed largely through the potentiation of GABA-mediated ion fluxes (Ransom and Barker, 1976; MacDonald and Barker, 1978; Choi et al., 1981; Haefely and Polc, 1986), the actions of these drugs on GABA-mediated 36Cl⁻ flux could be used to study BZ actions under various experimental conditions.

Animals chronically treated with BZ agonists become less responsive or tolerant to the drug (File, 1983; Gent and Haigh, 1983; Frey et al., 1984; Rosenberg and Chiu, 1985; Rosenberg et al., 1986). Some chronic BZ treatment protocols have been associated with a reduction in BZ binding sites (Rosenberg and Chiu, 1981a,b; Crawley et al., 1982; Sher et al., 1983; Tietz et al., 1986), an apparent shift of low-affinity state GABA binding sites into a higher affinity state (Gallager et al., 1984b, 1985), reduction in the ability of BZ to enhance GABA-mediated inhibition (Sher et al., 1983; Wilson and Gallager, 1987) and subsensitivity of dorsal raphe neurons to GABA (Gallager et al., 1984a). However, there have been contrasting reports concerning the ability of GABA to modulate BZ binding after chronic BZ treatments (reduced: Gallager et al., 1984a; enhanced: Stephens and Schneider, 1985). In some studies, no decrease in sensitivity to GABA was observed after chronic BZ treatments (Sher et al., 1983; Wilson and Gallager, 1987). In a study of rotational behavior induced by unilateral microinjection of muscimol or FZP into the pars reticulata of the substantia nigra, both

ABBREVIATIONS: ANOVA, analysis of variance; BZ, benzodiazepine; DFZP, desalkyflurazepam; DZP, diazepam, FZP, flurazepam; GABA, γ-aminobutyric acid; PB, pentobarbital.
tolerance to the BZ and reduced sensitivity to the GABA agonist were found, but differing time courses suggested the possibility of separate regulatory mechanisms (Tietz and Rosenberg, 1988).

In view of these contrasting results, this study investigated the acute and chronic effects of BZs on GABA-mediated responses by directly monitoring the influx of $^{36}$Cl$^{-}$ into freshly prepared rat brain microsacs from pooled rat cerebral cortex and hippocampus. The microsac preparation has been reported to be almost cell free (Allan and Harris, 1986a). Endogenous GABA has a negligible effect in this preparation (Harris and Allan, 1985; Yu et al., 1986) in contrast to synaptoneuroses (Schwartz et al., 1985). This makes the microsac preparation more accurate in measuring the GABA log dose-response curve.

**Methods**

**In Vivo Pretreatment of Rats**

Acute DZP treatment. Male Sprague-Dawley rats (Harlan, Hamilton, MI; 220–225 g) were treated with DZP (10 mg/kg p.o. in 0.5% Tween 80) 30 min before sacrifice. This DZP treatment was chosen because, in a previous study, it produced a brain level of BZ-like activity similar to that in rats chronically treated with FZP for 4 weeks, assayed by a radioligand binding technique (Tietz et al., 1986). Control rats were treated with 0.5% Tween 1 (1 ml/kg p.o.).

Chronic FZP treatment. Rats (initial weight 150–175 g; 280–310 g at sacrifice) were treated as previously described (Rosenberg and Chiu, 1981a). FZP, dissolved in 0.02% saccharin solution, was given as the only drinking water source for each rat. Based on the weight of each rat and on the volume consumed over the previous 24 hr, the drug concentration was adjusted to provide 100 mg/kg daily for the 1st week and 150 mg/kg daily for the subsequent 3 weeks. The doses actually received were very similar to those previously reported (Rosenberg and Chiu, 1981a). After 4 weeks, the rats were sacrificed at 0, 12, 24 or 48 hr or 7 days after stopping FZP treatment. The drug water was replaced with 0.02% saccharin for the drug-withdrawn rats. Control rats were given 0.02% saccharin and the same handling as treated rats.

**Preparation of Brain Microsacs**

Rats were killed by decapitation with a guillotine. The cerebral cortex and hippocampus (pooled weight about 0.9 g) were obtained on the GABA receptor desensitization. When preincubated with the membrane preparation for 15 min, DZP, DFZP, FZP and PB produced varying degrees of sensitivity to GABAergic agonists (results not shown), indicating that the effect of endogenous GABA on Cl$^{-}$ influx in this preparation is insignificant.

**Results**

**Effect of in vitro addition of BZs.** The BZs and PB were both shown to enhance GABA-gated Cl$^{-}$ influx into microsacs (figs. 1, 2 and 3). Midazolam (0.5 $\mu$M) produced a leftward parallel shift of the GABA log dose-response curve without significantly affecting the maximum response, whereas PB (20 $\mu$M) produced a leftward shift that was accompanied by an increase in maximum response of the GABA log dose-response curve (fig. 1). In the absence of added GABA, neither midazolam nor PB was able to mediate Cl$^{-}$ influx at the doses tested (results not shown).

In a recent study, the enhancement of muscimol-stimulated Cl$^{-}$ uptake by FZP in a rat brain synaptoneurosome preparation was identical regardless of whether the membrane was preincubated with FZP for 15 min (Morrow and Paul, 1988), indicating that FZP did not accelerate GABA receptor desensitization. When preincubated with the membrane preparation for 15 min, DZP, DFZP, FZP and PB produced varying degrees of sensitivity for Cl$^{-}$ uptake by FZP.
enhancement of 10 μM GABA-gated Cl⁻ influx (figs. 2 and 3). The effects of BZs were antagonized by Ro15-1788, indicating a specific interaction of BZs with BZ recognition sites on the GABA-BZ-Cl⁻ channel complex.

**Effects of acute BZ pretreatment.** Acute DZP pretreatment (10 mg/kg p.o.) enhanced GABA-gated Cl⁻ flux into the microsacs (fig. 4), with no effect on basal Cl⁻ influx (28.2 ± 1.9 vs. 28.3 ± 1.6 nmol/mg of protein; n = 10–12). Though the Cl⁻ influx caused by 10 μM GABA in these acutely treated animals was significantly larger than that of controls (30.5 ± 1.2 vs. 19.7 ± 1.7 nmol/mg of protein; n = 10–12, P < .05, Student’s t test), the enhanced influx in the treated animals was not reversed by the addition of 10 μM Ro15-1788 (33.9 ± 2.0 nmol/mg of protein; n = 10). The GABA log dose-response curve was shifted to the left, with no significant change in the maximum response or slope. This potentiation of the effect of 10 μM GABA (about 40% enhancement, fig. 4) was not as great as the in vitro effect of 1 μM DZP assayed with 10 μM GABA (70% enhancement, fig. 3). The positive modulation of BZs and PB of GABA-gated Cl⁻ influx was negligibly affected by acute in vivo pretreatment with DZP (fig. 3). The slight reduction in the drug enhancement of GABA-gated Cl⁻ influx was due to a larger denominator (i.e., increased 10 μM GABA-gated Cl⁻ influx in the absence of added drug; see fig. 4). If the same denominator for the nonpretreated experiment were used, there would be an equal amount of, or even a slight increase in, the net GABA-gated Cl⁻ flux rather than a slight reduction. The effects of acute DZP pretreatment shown in figure 3 were not statistically significant.

**Effect of chronic BZ treatment.** Similar to the effects of in vitro addition of midazolam or acute pretreatment with DZP, a significant, parallel shift to the left of the GABA log dose-response curve was observed after 4-week FZP treatment in the nonwithdrawn rats (fig. 5). The maximum response to GABA was increased, though this was significant only at 12 hr after chronic treatment (fig. 6). The Cl⁻ influx induced by 10 μM GABA in the nonwithdrawn rats was significantly greater than that in controls (31.7 ± 2.7 vs. 19.7 ± 1.7 nmol/mg of protein; n = 9–12, P < .05, Student’s t test). Addition of Ro15-1788 had no effect on the GABA-gated Cl⁻ influx (31.7 ± 2.4 nmol/mg of protein; n = 9).

After 4 weeks of FZP treatment, the enhancement of GABA-gated Cl⁻ influx by the in vitro addition of 1 μM DZP, 1 μM DFZP and 100 μM FZP was reduced compared with control animals (fig. 7), showing tolerance. The greatest reduction in BZ effects was in tissue prepared from nonwithdrawn rats. There appeared to be some recovery in tissues taken from rats 2 days after stopping chronic FZP treatment, and even more recovery by 7 days (fig. 7). Similar results were found with PB (fig. 8), showing cross-tolerance to the barbiturate. These data were analyzed by ANOVA, which showed significant differences between the test drugs used (F = 12.5, P < .0001) and significant differences between days (i.e., control and 0, 2 and 7 days after chronic treatment; F = 11.5, P < .0001). There was no significant interaction between these factors (P > .50). The data for each drug were then analyzed by one-way ANOVA and multiple-range testing by the method of least-square differences. There was a significantly reduced effect on GABA-gated Cl⁻ influx for 1 μM DZP (F = 4.58, P < .01), 100 μM FZP (F = 3.18, P < .04) and 20 μM PB (F = 5.69, P < .01). The differences

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Fig. 2. Effect of in vitro addition of BZs on 10 μM GABA-gated Cl⁻ influx into pooled hippocampal and cortical microsacs. BZs were preincubated for 15 min in the absence (open bar; n = 12), and presence (striped bar; n = 12) of 10 μM Ro15-1788. GABA-gated Cl⁻ influx was assayed for 5 sec. All drugs were assayed at a final concentration of 0.02% dimethylsulfoxide. Dimethylsulfoxide at 0.1 to 0.4% had no effect on either the basal or 10 μM GABA-gated Cl⁻ influx (results not shown). An asterisk represents significant antagonism by Ro15-1788 (P < .01; pooled two-tailed Student’s t test). Vertical lines show S.E.M.

Fig. 3. Enhancement by BZs and PB of GABA-gated Cl⁻ flux in control rats (open bars; n = 12) and acutely pretreated rats (striped bars; n = 10) given DZP, 10 mg/kg p.o., 30 min before sacrifice. All drugs were preincubated with the microsacs preparation for 15 min, and GABA-gated Cl⁻ influx was assayed for 5 sec. All drugs were assayed at a final concentration of 0.2% dimethylsulfoxide. Vertical lines are S.E.M.

Fig. 4. Effect of acute pretreatment of rats with DZP, 10 mg/kg p.o., 30 min before sacrifice (n = 8) on GABA-gated chloride ion flux. This treatment produces similar brain BZ-like activity as chronic FZP treatment. Control rats (n = 8) were treated with the vehicle, 0.5% Tween 80, 1 mg/kg p.o. The GABA dose-response curve was shifted to the left after acute DZP exposure with no change in the maximum response. Values shown are means ± S.E.M.
were significant only in tissues prepared from nonwithdrawn rats (i.e., 0 days off of chronic treatment) and not in brain tissues taken 2 or 7 days after chronic treatment. There was no change in GABA-gated Cl⁻ flux in the presence of 10 μM Ro15-1788 (F = 0.07, P > .9). Though there appeared to be tolerance to DFZP (fig. 7), the difference was not significant by ANOVA (F = 2.08, P > .1). However, a specific planned comparison of the data at time 0 (nonwithdrawn rats) with the control data revealed a significant difference (P < .05 by t test), suggesting that tolerance was indeed present.

Fig. 6. Effect of 4 weeks of FZP treatment on GABA-gated Cl⁻ flux. Rats were treated with FZP as in figure 5 and then withdrawn from FZP by replacing the drug solution with 0.02% saccharin solution for either 12 (n = 6) or 48 (n = 5) hr before they were decapitated. Control rats (n = 10) were treated with 0.02% saccharin water for the same duration. Values shown are means ± S.E.M.

Discussion

The main finding of this study was that chronic but not acute BZ treatment caused tolerance to the actions of BZs and PB to enhance GABA-gated Cl⁻ influx into brain microsacs. Though BZs and PB both potentiated GABA-gated Cl⁻ influx, as has been previously reported (Allan and Harris, 1986b; Schwartz et al., 1986a,b; Obata and Yamamura, 1986; Morrow and Paul, 1988), the dose-response data (fig. 1) show that they do so by different mechanisms, as was found in electrophysiological studies (Study and Barker, 1981).

One possible drawback of the 15-min preincubation of the microsacs with BZs or PB was the possibility that enhanced desensitization of the GABA effect might occur. Desensitization of muscimol-stimulated Cl⁻ uptake by PB (Schwartz et al., 1986b) and ethanol (Suzdak et al., 1986) in once-washed rat
brain synaptoneurosomes has been reported. On the other hand, the enhancement of muscimol-stimulated Cl\(^-\) uptake by FZP in twice-washed rat brain synaptoneurosomes was identical regardless of whether the membrane was preincubated with FZP for 15 min (Morrow and Paul, 1988). Either desensitization of GABA agonist-induced Cl\(^-\) uptake is ligand specific or additional washing of the membrane removes sufficient endogenous GABA or other substances that may be involved in the desensitization process. In the present study, we have used a twice-washed membrane microsac preparation. The basal Cl\(^-\) influx of this preparation was not affected by GABA antagonist, bicuculline, or GABA uptake inhibitor, nipecotic acid (Harris and Allan, 1985; Yu et al., 1986; present study), indicating that endogenous GABA did not play a significant role in mediating Cl\(^-\) influx. In conjunction with the recent report by Morrow and Paul (1988), it is very likely that BZs did not accelerate GABA receptor desensitization in the present study.

In this study, it was necessary to consider the possibility that the effects observed might be due to residual drug remaining in brain microsacs after acute or chronic treatment. Washing the microsacs during tissue preparation should have reduced the amount of residual BZ. In fact, residual BZ apparently had no effect because the enhanced GABA-gated Cl\(^-\) influx in the acutely pretreated rats as well as the enhanced GABA-gated Cl\(^-\) flux in chronically treated rats was not affected by Ro15-1788. Thus, the elevated GABA response was not due to the continued presence of BZ at the receptor site.

The concentrations of BZs needed to enhance GABA-gated Cl\(^-\) influx were higher than the apparent dissociation constants measured at 0°C in the in vitro binding studies (Braestrup and Squires, 1978; Tyma et al., 1984). The difference is likely due to the rather crude membrane preparation and the higher temperature used in the present study. The crude membrane preparation will undoubtedly absorb more BZs to non-receptor sites, reducing their effective concentrations. Higher temperature is known to decrease the affinity of many ligands, including BZs. However, the rank order of potency of the BZs (DZP = DPZP > FZP > Ro15-1788) was as expected from electrophysiologic studies (Chan and Farb, 1985).

The effects of acute DZP pretreatment indicated that the GABA recognition site-CI\(^-\) channel complex may be a site of acute and rapid modulation. The mechanism for this regulation is not clear, though other data also suggest rapid modulation of the GABA receptor-CI\(^-\) channel. For example, an increase in the number of GABA binding sites has been reported after an acute dose of DZP in mice (Ferrero et al., 1984). Also, exposure of rats to the stress of a swim test was reported to cause an increased potency and efficacy of CI\(^-\) to potentiate \(^{[3H]}\)flunitrazepam binding, and an increased number of \[^{[3H]}\]\text{-butylbicyclophosphorothionate binding sites (Havoundjian et al., 1986). In the present study, DZP pretreatment had no effect on the subsequent ability of BZs, added to the assay, to enhance GABA-gated Cl\(^-\) influx (fig. 3). These results indicated that acute DZP treatment apparently did not alter the sensitivity of BZ recognition sites. These observations suggest that BZ and GABA recognition sites can be differentially regulated.

As was found with the acute DZP treatment, there was a shift to the left of the GABA log dose-response curve (in the absence of in vitro addition of BZs) in brain tissue from nonwithdrawn rats after a standard 4-week FZP treatment (fig. 5). Both acute DZP pretreatment and chronic FZP treatment result in a comparable BZ-like activity in the brain (Rosenberg et al., 1985; Tietz et al., 1986). The enhanced GABA effect in the nonwithdrawn rats was not reversed by Ro15-1788. Therefore, as discussed above, this increased GABA effect was not due to the continued presence of BZ at the receptor. In preliminary experiments, using nonsaturating concentrations of \(^{[3H]}\)GABA, we have found an increase in GABA binding after the 4-week FZP treatment (unpublished results). Therefore, the shift in the GABA log dose-response curve may have resulted from an increase in GABA binding observed after chronic FZP treatment. Increased GABA binding was also observed in rats chronically treated with DZP (Gallager et al., 1984b, 1985) and mice chronically treated with clonazepam (Marangos and Crawley, 1982). In another study (Stephens and Schneider, 1985), tolerance produced in mice by nine daily injections of 5 mg/kg of DZP was associated with an increased effect of GABA to potentiate \(^{[3H]}\)ormetazepam binding. Despite the increased GABA binding and ability to potentiate BZ binding, subsensitivity to GABA (Gallager et al., 1985) and muscimol (Tietz and Rosenberg, 1988) was present in tolerant rats. Because GABA-gated Cl\(^-\) influx was increased in tolerant rats (figs. 5 and 6), the subsensitivity to GABA and muscimol observed in intact animals might be due to an alteration of neuronal function subsequent to activation of the GABA-gated Cl\(^-\) channels.

In contrast to the GABA response, the enhancement of GABA-gated Cl\(^-\) influx by BZs was reduced in the chronically treated rats, indicating tolerance to BZs. The potentiating effect of PB was also decreased, demonstrating cross-tolerance. Tolerance to the effect of BZs on GABA-gated Cl\(^-\) influx was no longer significant 2 days after stopping FZP treatment, similar to the reversal of tolerance to the ataxia produced by large doses of BZs tested in animals given the same chronic FZP treatment (Rosenberg and Chiu, 1981a; Rosenberg et al., 1983). In contrast, tolerance to the antipentylenetetrazol seizure activity reversed between 4 and 7 days post-chronic treatment (Rosenberg et al., 1985). It is possible that the results of the present study are related to tolerance to BZ-induced ataxia, but it seems unlikely that these findings are related to tolerance that lasts longer. The recovery from cross-tolerance to PB was also rapid. PB enhancement of GABA-gated Cl\(^-\) influx was no longer significantly less than control value 2 days after ending the FZP treatment. In contrast, tolerance measured by the ataxia produced by PB did not return to control until 7 days after stopping FZP treatment (Rosenberg et al., 1983). Thus, cross-tolerance to PB, measured by ataxia, and tolerance to the antipentylenetetrazol effect of BZs cannot be ascribed directly to changes in GABA receptor number or function. Tolerance seen in intact animals must be mediated in part by changes in neuronal function that occur subsequently to activation of the BZ-GABA-CI\(^-\) channel. However, the present results do not rule out the possibility that tolerance, measured by BZ enhancement of GABA-gated Cl\(^-\) flux, might persist longer in specific brain regions, but these would be obscured by the use of large brain areas for assay.

In conclusion, the present experiment showed that, using an in vitro method for studying GABA-gated Cl\(^-\) flux, 1) the actions of BZs and PB are functionally linked to the GABA receptor-CI\(^-\) channel complex but not by identical mechanisms, 2) acute treatment with DZP does not produce tolerance to BZs or PB, whereas chronic FZP treatment produces tolerance to BZs and cross-tolerance to PB and 3) both acute and chronic BZ treatments produce a supersensitivity to GABA that is not reversed by Ro15-1788. The present study suggests that the
function of the GABA recognition site-CI channel and BZ enhancement of GABA response can be separately modulated.

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References


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