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AUGMENTATION OF $GABA_A$ RECEPTOR FUNCTION BY CHRONIC EXPOSURE TO GABA-NEUTRAL AND GABA-NEGATIVE BENZODIAZEPINE LIGANDS IN CULTURED CORTICAL NEURONS

LAWRENCE G. MILLER,* JACK HELLER, MONICA LUMPKIN, CHERYL L. WEILL, DAVID J. GREENBLATT and RICHARD I. SHADER

Division of Clinical Pharmacology, Departments of Psychiatry and Pharmacology, Tufts University School of Medicine and New England Medical Center, Boston, MA; and Departments of Neurology and Anatomy, LSU Medical Center, New Orleans, LA, U.S.A.

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Abstract—Chronic benzodiazepine agonist administration may lead to decreases in γ-aminobutyric $acid_A$ ($GABA_A$) receptor binding and function, but little information is available concerning chronic GABA-neutral or GABA-negative benzodiazepine exposure. We evaluated effects of chronic exposure to flumazenil (Ro15-1788) and FG 7142 (*N*-methyl-β-carboline-3-carboxamide) on GABA-dependent chloride uptake in chick cerebral cortical neurons in primary culture. Acute flumazenil treatment (1 μM) had no effect on chloride uptake, but uptake was increased after 2 days of exposure. Similar increases were observed after 4 and 10 days. Flumazenil, 0.1 μM, had no effect after 10 days, and a 10 μM concentration had a similar effect as the 1 μM concentration. Acute FG 7142 (1 μM) decreased chloride uptake, but uptake was increased markedly after 2, 4, and 10 days of treatment. No effect was observed after treatment for 10 days with 0.1 μM, but a 10 μM concentration showed similar enhancement to the 1 μM concentration. Concurrent treatment with 0.3 μM flumazenil which did not affect chloride uptake and 1 μM FG 7142 for 10 days substantially attenuated the effects of FG 7142, suggesting that FG 7142 effects are mediated at the benzodiazepine site. Benzodiazepine receptor binding was increased in cultures treated for 10 days with 1 μM flumazenil or FG 7142, with an increase in receptor number in both cases but no change in apparent affinity. Neither flumazenil nor FG 7142 (1 μM for 10 days) altered GABA-independent chloride uptake, total cellular protein, protein synthesis or degradation, or neuronal survival. These results indicate that both chronic GABA-neutral and GABA-negative benzodiazepine exposures in cultured cortical neurons lead to increases in GABA-dependent chloride uptake and benzodiazepine binding. Effects of GABA-negative benzodiazepine exposure appear to be greater than those observed with GABA-neutral benzodiazepine exposure.

Several types of ligands have been reported to interact with the benzodiazepine site on the $GABA_A$† receptor complex. These include classical benzodiazepines, such as diazepam or lorazepam, and the more recently developed triazolobenzodiazepines such as alprazolam and triazolam. These compounds have little direct effect on the $GABA_A$ receptor-coupled chloride channel, but rather exert their effects by allosteric enhancement of binding to the GABA site (GABA-positive or agonists) [1]. In addition, several types of compounds have been reported to bind to the benzodiazepine site, but to have little intrinsic activity [2, 3]. These compounds, such as the imidazobenzodiazepine flumazenil (Ro15-1788), compete for binding with agonist compounds but have no effect on the GABA site or the chloride channel (GABA-neutral or antagonists). A third class of compounds has also been described with actions opposite to the agonist compounds; that is, these compounds decrease GABA binding and thus chloride transport (GABA-negative or inverse agonists). GABA-negative benzodiazepines include some β-carbolines and a more stable amide derivative, FG 7142 [4].

The acute effects of benzodiazepine receptor ligands on $GABA_A$-related chloride transport have been characterized in some detail, but less information is available concerning effects of chronic administration of these compounds on $GABA_A$ receptor function. Chronic administration of GABA-positive benzodiazepine has been reported to lead to behavioral tolerance and a decrease in GABA-related chloride transport in several experimental systems [5, 6]. With regard to GABA-neutral ligands, several studies of chronic flumazenil administration reported increases in motor activity, benzodiazepine binding and, in one study, $GABA_A$ receptor function [7, 8]. Chronic administration of GABA-negative ligands has been reported to augment convulsant effects of inverse agonists and to diminish anticonvulsant effects of agonists [9–12].

Primary culture of neurons offers controlled conditions in which to evaluate effects of chronic drug exposure, avoiding problems of drug delivery and metabolism [13]. A method to assess $GABA_A$ receptor function in intact neurons has been developed

* Correspondence: Dr. Lawrence G. Miller, Division of Clinical Pharmacology, Box 1007, 171 Harrison Ave., Boston, MA 02111.

† Abbreviations: $GABA_A$, γ-aminobutyric $acid_A$; FG 7142, *N*-methyl-β-carboline-3-carboxamide; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; and SDS, sodium dodecyl sulfate.

recently [14], and we have reported marked decreases in GABA-related chloride uptake in cells chronically treated with the GABA-positive benzodiazepine clonazepam [13]. In the present study, we employed these techniques to evaluate the effects of a chronic GABA-neutral benzodiazepine (flumazenil) and a GABA-negative benzodiazepine (FG 7142) on $GABA_A$ receptor function.

MATERIALS AND METHODS

Embryonated White Leghorn eggs were obtained from Truslow Farms (Chestertown, MD) and were maintained in a humidified, forced-draft incubation at 37.5°. [$^{36}Cl^-$] (sp. act. 12.5 mCi/g), [^{3}H]flunitrazepam (sp. act. 71 Ci/mmol), and [^{3}H]ouabain (sp. act. 15 Ci/mmol) were obtained from New England Nuclear (Boston, MA). Muscimol was purchased from the Sigma Chemical Co. (St. Louis, MO). FG 7142 was purchased from Research Biochemicals (Natick, MA). Flumazenil was provided by Hoffmann–LaRoche (Nutley, NJ). All other reagents were obtained from standard commercial sources.

Tissue culture. Cortices were obtained from 8-day-old chick embryos staged according to the series of Hamburger and Hamilton [15]. The tissue was minced into 1-mm pieces and incubated in Puck's D_1G medium containing 0.5% trypsin for 30 min at 37° without stirring. The tissue was collected by centrifugation, resuspended in 5% MEM (Eagle's Minimum Essential Medium, containing 10% heat-inactivated horse serum, 5% chick embryo extract, 2 mM glutamine, 50 units/mL penicillin, and 50 μg/mL streptomycin) and triturated using a fire-polished pasteur pipet. Cells were placed on collagenized vinyl plastic coverslips in the presence of cytosine arabinoside (ara-C, 10^{-5} M) at a density of 10^6 cells per 100-mm dish containing six coverslips. Cultures were maintained in an atmosphere of 5% CO_2/95% humidified air at 37°. Cultures were fed every other day with fresh 5% MEM without ara-C, containing the appropriate concentration of flumazenil, FG 7142, or vehicle.

Drug administration. Flumazenil and FG 7142 were dissolved in ethanol and diluted with saline to less than 1% ethanol. Final concentration in culture medium was less than 0.01% ethanol. Control cultures were treated with vehicle alone. Cultures were treated with each drug at 1 μM for 1 hr and 2, 4, and 10 days, and at 0.1 and 10 μM for 10 days. Treatments for 2 and 4 days were administered at days 8–10 and 6–10 of culture respectively.

[$^{36}Cl^-$]*uptake.* Uptake of labeled chloride was performed using a modification [13] of the method of Thampy and Barnes [14]. All uptake experiments were performed after 10 days of culture. Briefly, coverslips were removed from tissue culture medium and incubated at 37° for 5 min in fresh Dulbecco's modified Eagle's medium. Coverslips were then rinsed in HEPES-buffered saline at 22° for 5 sec and then drained and transferred to high potassium HEPES-buffered saline (96 mM NaCl, 40 mM KCl, 1.4 mM $MgCl_2$, 1.2 mM $CaCl_2$, 1 mM NaH_2PO_4, 20 mM HEPES, pH 7.4), containing [$^{36}Cl^-$] (5–10 μCi/mL) and muscimol, 1–100 μM. After a 10-sec interval, uptake was terminated by transfer of coverslips to 600 mL of an ice-cold stop solution for 8 sec. Coverslips were then placed in 0.5 M NaOH at 22° for 30 min. After removal of an aliquot for protein determination, the solution was neutralized with glacial acetic acid and counted by conventional scintillation spectrometry. Protein concentrations were determined by the method of Simpson and Sonne [16].

Control experiments previously reported [13] indicate that the 10-sec interval used in these studies represents uptake rather than equilibration of chloride within an intracellular compartment. In addition, GABA-dependent but not GABA-independent uptake is blocked by the chloride channel antagonist picrotoxinin, indicating the specificity of the assay for $GABA_A$ receptor-mediated transport.

Morphological analysis. To assess neuronal survival in cultures treated with flumazenil and FG 7142, cells were plated at one-half density and maintained as above. After 10 days, ten high-power fields from treated and control cultures were examined and non-neuronal cells counted.

Protein synthesis and degradation. For cellular protein synthesis, cultures were incubated with [^{35}S]methionine (20 nM, 30 min at 37°). Cultures were then washed to remove unincorporated radioactivity. SDS/Nonidet P-40/urea (0.2%/2%/8 M) was added, and aliquots were removed. Trichloroacetic acid (TCA) was added to a final concentration of 10% and the samples were incubated at 4° for 30 min. Samples were filtered on Whatman GF/B filters which were washed twice with Tris–HCl (pH 7.4 at 4°). Filters were counted by scintillation spectrometry. For cellular protein degradation, cultures were labeled with [^{35}S]methionine (2 nM, 24 hr at 37°), washed with medium containing 2 mM methionine to prevent further incorporation of label, and incubated for an additional 6 hr with fresh medium. Aliquots were removed and TCA was added to a final concentration of 10%. Samples were incubated at 4° for 30 min followed by centrifugation (15,000 *g*), and supernatant fractions were counted by scintillation spectrometry.

[^{3}H]*Ouabain binding.* Binding was performed as described by Hauger *et al.* [17]. Cells were scraped from dishes and washed with 50 mM Tris–HCl (pH 7.4 at 4°), 1000 *g* for 10 min at 4°. Cells were homogenized by hand (5 strokes) in a Teflon–glass homogenizer, and the homogenate was centrifuged at 15,000 *g* for 10 min at 4°. Membranes were washed with Tris–HCl (45,000 *g* for 10 min at 4°) and resuspended gently in 100 mM Tris–HCl, 10 mM $MgCl_2$, and 100 mM NaCl, pH 7.4, at 22°. Binding was performed in duplicate or triplicate using 25 nM [^{3}H]ouabain, and 5 mM ATP to determine specific binding. Samples were incubated for 60 min at 22°, and then filtered on Whatman GF/B filters. Filters were washed twice with cold buffer and counted by scintillation spectrometry.

Benzodiazepine binding. To remove residual clonazepam or vehicle, membranes were prepared as described above. After washing, membranes were suspended in 10 mM PMSF/10 mM EDTA (0.25 mL/dish) and dialyzed using 15,000 mol. wt

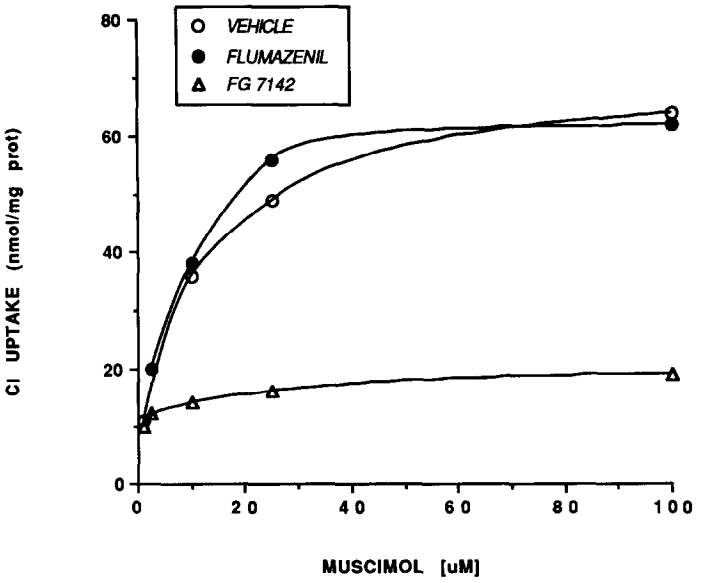

Fig. 1. Effects of acute flumazenil and FG 7142 exposure on muscimol-stimulated chloride uptake. Neurons at day 10 of culture were exposed for 1 hr to flumazenil or FG 7142 (1 μM), and chloride uptake was determined. Results are means from three separate experiments performed in duplicate.

cutoff tubing for 48–72 hr at 4° in 4 L of 25 mM potassium phosphate buffer, pH 7.4. Dialysate was changed twice (total dialysis volume 12 L). Membranes were then washed with 50 mM Tris–HCl as above, and resuspended in this buffer. Binding was performed in duplicate or triplicate using [^{3}H]flunitrazepam (0.1 to 5 nM). An additional set of samples was treated with 5 μM flurazepam to determine nonspecific binding. Samples were incubated for 45 min at 4°, and then filtered on Whatman GF/B filters. Filters were washed twice with buffer and counted by scintillation spectrometry. Data analysis was performed using the EBDA programs.

Data analysis. In chloride uptake experiments, non-GABA-related uptake was subtracted from total uptake to yield GABA-related uptake. For analysis, data from two to four experiments performed in triplicate or quadruplicate were pooled, and analysis of variance was used with Dunnett's correction. For other experiments, data were analyzed by analysis of variance with Dunnett's correction or by Student's *t*-test for two groups.

RESULTS

Flumazenil. Treatment of cultures with 1 μM flumazenil for 1 hr had no effect on muscimol-stimulated chloride uptake compared to cultures treated with vehicle alone (Fig. 1). After 2 days of treatment, chloride uptake at muscimol concentrations of 10, 25 and 100 μM was increased substantially in flumazenil-treated cultures compared to cultures treated with vehicle alone (Fig. 2). Similar results were obtained after 4 and 10 days of flumazenil treatment, and no differences were observed among these three time points. Maximal chloride uptake was unchanged from controls after 0.1 μM flumazenil, for 10 days (Fig. 3). After treatment with 10 μM flumazenil for 10 days, maximal uptake was increased compared to controls but was not significantly different from cultures treated with 1 μM flumazenil. Chronic flumazenil treatment did not affect GABA-independent chloride uptake (data not shown).

FG 7142. Treatment of cultures with 1 μM FG 7142 for 1 hr substantially decreased chloride uptake at muscimol concentrations of 2.5, 10, 25, and 100 μM (Fig. 1). After 2 days of treatment, chloride uptake at muscimol concentrations of 2.5, 10, 25, and 100 μM was increased markedly compared to vehicle-treated control cultures (Fig. 4). Similar increases were observed after 4 and 10 days of treatment, and no differences were observed among these three time points. Maximal chloride uptake was unchanged compared to controls after 0.1 μM FG 7142 for 10 days (Fig. 5). After treatment with a 10 μM concentration for 10 days, uptake was increased markedly compared to controls but was not changed significantly from 1 μM FG 7142. Chronic FG 7142 treatment did not affect GABA-independent chloride uptake (data not shown).

Flumazenil/FG 7142. To determine whether effects of FG 7142 were mediated at the benzodiazepine site, cultures were treated concurrently for 10 days with 1 μM FG 7142 and 0.3 μM flumazenil. This concentration of flumazenil alone did not affect GABA-dependent chloride uptake. Concurrent treatment with flumazenil significantly ($P < 0.05$) attenuated the effects of FG 7142 on maximal GABA-dependent chloride uptake (Fig. 6), with no effect on GABA-independent chloride uptake.

Benzodiazepine binding. To assess benzodiazepine binding after chronic flumazenil and FG 7142 treatment, membranes from cultures treated for 10 days with flumazenil or FG 7142 (1 μM) or vehicle

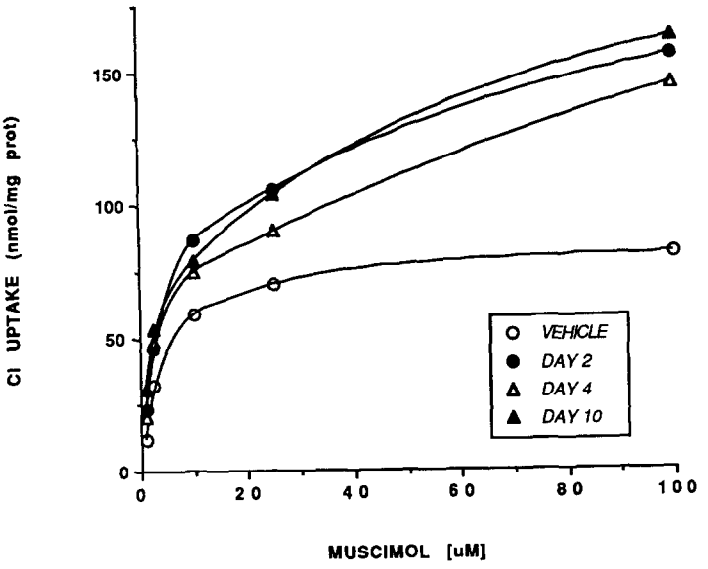

Fig. 2. Effects of chronic flumazenil treatment on muscimol-stimulated chloride uptake. Neurons were exposed to flumazenil (1 μM) for 2 days (culture days 8–10), 4 days (culture days 6–10) or 10 days (culture days 1–10), and chloride uptake was determined at day 10. Results are means from three experiments performed in duplicate for vehicle and day 4, and four experiments for days 2 and 10.

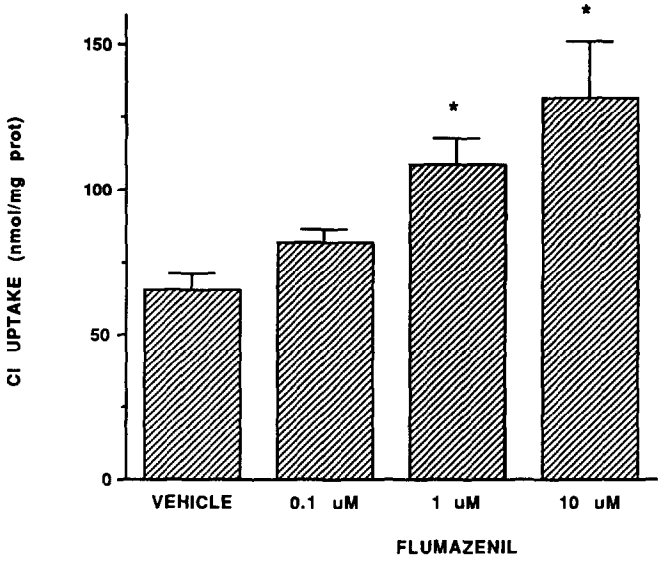

Fig. 3. Effects of flumazenil concentration on muscimol-stimulated chloride uptake after 10 days of treatment. Neurons were exposed for 10 days of culture to flumazenil (0.1, 1, 10 μM) and chloride uptake was determined at 10 days. Results are means ± SE, N = 3 experiments for vehicle, 0.1, and 10 μM, N = 4 experiments for 10 μM. Key: (*)P < 0.05 vs vehicle and 0.1 μM.

were prepared and dialyzed extensively to remove residual drug. Rosenthal–Scatchard analyses were then performed using [^{3}H]flunitrazepam (Table 1). For both flumazenil and FG 7142, apparent affinity (K_d) was unchanged, but the number of binding sites (B_{max}) was increased significantly compared to controls. Results were not significantly different between the two drug treatments (P = 0.07).

Effects on neuronal protein synthesis and survival. Chronic administration of flumazenil or FG 7142 might affect neuronal survival, protein synthesis or the presence of non-neuronal cells. With regard to total neuronal protein, protein determinations in coverslips treated with flumazenil or FG 7142 (1 μM, 10 days) were similar in a large number of experiments. For three representative experiments: vehicle, 2.17 ± 0.14 μg/mL; flumazenil, 1.81 ± 0.18 μg/mL; and FG 7142, 2.17 ± 0.09 μg/

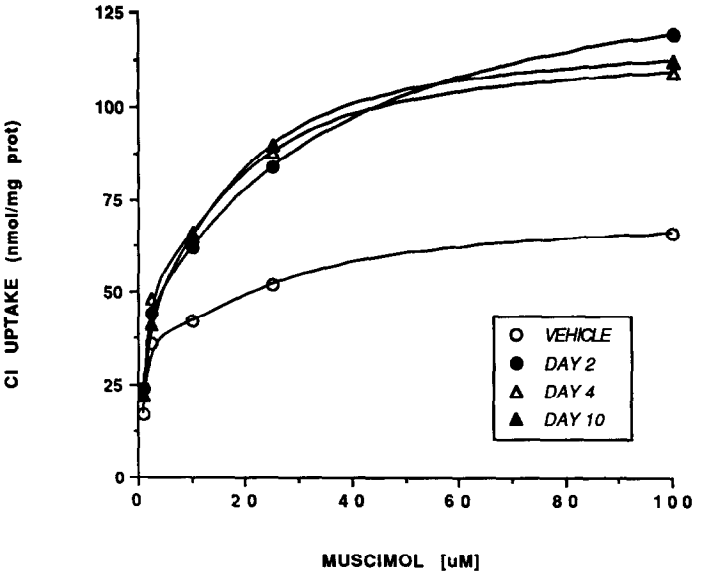

Fig. 4. Effects of chronic FG 7142 treatment on muscimol-stimulated chloride uptake. Neurons were exposed to FG 7142 (1 μM) for 2 days (culture days 8–10), 4 days (culture days 6–10) and 10 days (culture days 1–10), and chloride uptake was determined at day 10. Results are means from three experiments performed in duplicate for vehicle, day 2, and day 4, and four experiments for day 10.

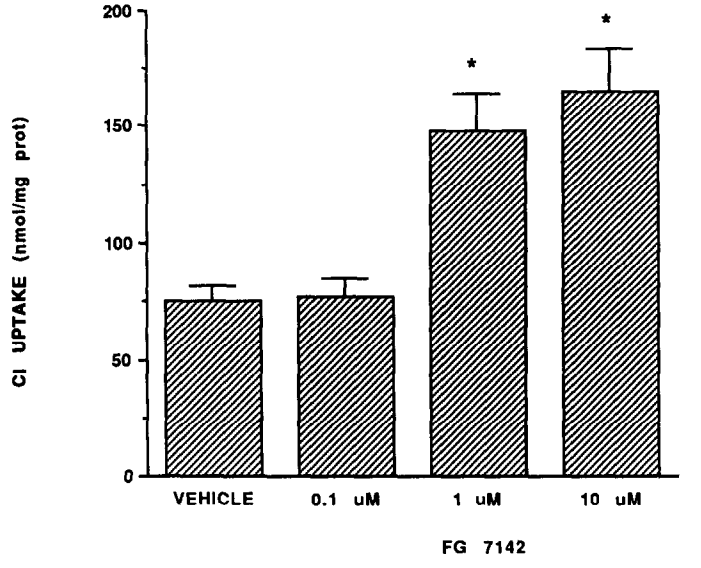

Fig. 5. Effects of FG 7142 concentration on muscimol-stimulated chloride uptake after 10 days of treatment. Neurons were exposed for 10 days to FG 7142 (0.1, 1, and 10 μM), and chloride uptake was determined at 10 days. Results are means ± SE, N = 3 experiments for each dose. Key: (*) $P < 0.05$ vs vehicle and 0.1 μM.

mL; mean ± SE, N = 12 in each experiment. Rates of protein synthesis and degradation were also similar in cultures treated with chronic flumazenil or FG 7142 and vehicle-treated controls (Synthesis: control, 100 ± 11%; flumazenil, 112 ± 6%; FG 7142, 92 ± 12%; Degradation: control, 100 ± 17%; flumazenil, 107 ± 10%; FG 7142, 113 ± 13%; mean ± SE). With regard to neuronal survival, no changes in the percentage of non-neuronal cells were observed in ten representative microscopic fields in cultures treated with flumazenil or FG 7142, 1 μM for 10 days, or vehicle: vehicle, 16.4 ± 0.8; flumazenil, 17.1 ± 1.3; FG 7142, 18.0 ± 1.2 (mean ± SE). Both vehicle- and drug-treated cultures contained greater than 95% neuronal appearing cells. Binding of [^{3}H]ouabain, which appears to be highly specific for neurons, was also similar in cultures treated with flumazenil or FG 7142 and controls (percent control

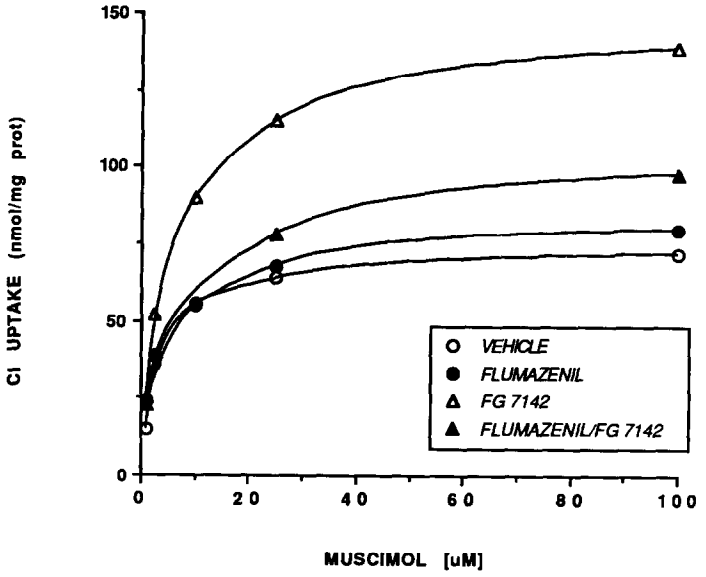

Fig. 6. Effects on concurrent administration of flumazenil and FG 7142 on muscimol-stimulated chloride uptake. Neurons were treated with 0.3 μM flumazenil and 1 μM FG 7142 or vehicle for 10 days. Results are means of three separate experiments performed in triplicate or quadruplicate.

Table 1. Effect of chronic flumazenil and FG 7142 treatment on benzodiazepine binding

Treatment	K_d (nM)	B_{max} (fmol/mg protein)	N
Vehicle	1.40 ± 0.17	404 ± 60	6
Flumazenil	1.42 ± 0.20	566 ± 62*	3
FG 7142	1.39 ± 0.09	815 ± 119†	3

Cultures were treated for 10 days with vehicle, 1 μM flumazenil or 1 μM FG 7142. Cells were then homogenized and dialyzed extensively to remove drug prior to binding studies using [^{3}H]flunitrazepam. Results are means ± SE.
* P = 0.05.
† P = 0.02.

binding: flumazenil, 108 ± 9; FG 7142, 98 ± 8; mean ± SE, N = 6 for flumazenil, FG 7142, and controls).

DISCUSSION

Chronic benzodiazepine administration may have the capacity to modulate the entire $GABA_A$ receptor complex. Numerous studies demonstrate the development of behavioral tolerance during chronic GABA-positive benzodiazepine administration [18], and a number of studies indicate accompanying neurochemical alterations. In particular, several recent studies reported decreased benzodiazepine binding in several brain regions after chronic lorazepam or flurazepam administration [5, 19], and two studies reported decreased GABA-dependent chloride uptake in cortex after chronic lorazepam and diazepam administration [5, 6]. Thus, despite the fact that benzodiazepines act indirectly by allosteric modulation of GABA binding, chronic benzodiazepine administration appears to alter both structure and function of the $GABA_A$ receptor.

Limited information is available concerning chronic administration of a GABA-neutral benzodiazepine. At commonly used doses, the imidazobenzodiazepine flumazenil appears to have GABA-neutral properties, although some evidence indicates GABA-negative effects after acute high dose administration [20]. After chronic administration, several studies reported increases in motor activity, two studies reported increases in benzodiazepine binding, and one study found an accompanying increase in GABA-dependent chloride uptake [7, 8, 21]. Thus, most available data indicate that chronic GABA-neutral benzodiazepine administration produces effects opposite to those observed after chronic GABA-positive benzodiazepine administration. In addition, some behavioral evidence supports enhanced GABA-negative effects after chronic flumazenil treatment [20].

Although data are also limited, there is behavioral evidence that chronic FG 7142 administration leads to enhanced GABA-negative effects. In particular, chronic administration augments convulsant effects of [4, 10, 11, 22], and conversely decreases [10–12] anticonvulsant effects of GABA-positive ligands. There appears to be little effect on GABA-neutral ligand activity. One study reported no change in benzodiazepine binding after chronic FG 7142 treatment [23], whereas other groups found decreased effects of GABA agonists [24], decreased GABA-shift [25] and decreases in GABA-dependent chloride uptake [26, 27].

The results of the present study are consistent with prior studies concerning chronic flumazenil administration. As previously reported in tissue preparations

[28], acute flumazenil had no effect on GABA-dependent chloride uptake. However, after 2 days of flumazenil administration chloride uptake was increased substantially, and this change persisted during treatment for 4 and 10 days. The number of benzodiazepine binding sites was also increased at 10 days. It is unlikely that these effects could be due to persistence of flumazenil during uptake determination, since no alteration in uptake would be expected. In addition, toxicity of flumazenil to neurons might be expected to have opposite results, and in any case no change in total protein, protein synthesis or degradation, or neuronal survival was observed during drug administration. These data are similar to results obtained during treatment of intact animals, in which enhanced chloride uptake and benzodiazepine binding were also observed [8].

With regard to FG 7142, results of acute administration are similar to those observed in tissue preparations [28], indicating decreased chloride uptake. However, chloride uptake was increased markedly after 2 days of FG 7142, and increases persisted during 4 and 10 days of treatment. Benzodiazepine binding sites were also increased at 10 days. Neither persistence of FG 7142 during the assay nor neuronal toxicity would be expected to produce these effects, and evidence for toxicity was not observed. Our results are thus in contrast to two studies which reported a decrease in chloride uptake after chronic FG 7142, although these studies involved intermittent injection rather than continuous administration, and assays were performed several days after drug discontinuation [27, 28]. Our prior studies using benzodiazepine agonists indicate the possibility of markedly different effects on neurochemical parameters before and after discontinuation of chronic drug treatment [5, 29]. In addition, preliminary observations involving continuous infusion of FG 7142 indicate increases in $GABA_A$ receptor binding during chronic administration [30].

The drug concentrations used in this study were based on prior studies with GABA-positive benzodiazepines such as clonazepam [13]. The presence of a concentration–response effect for both flumazenil and FG 7142, as noted above, indicates that this range is appropriate for studies in culture. In addition, prior studies indicate that chronic flumazenil administration in mice at doses that produce receptor upregulation and accompanying neurochemical changes produces cortex concentrations of approximately 15 ng/g [8]. If the drug is assumed to be distributed homogeneously, and 1 g of tissue is analogous to 1 mL, then the 1 μM concentration used in the present study is approximately five to ten times greater than molar concentrations expected in *in vivo* studies. In view of the greater concentrations of drug required to affect cultured or dissociated neurons, the concentrations used in this study are likely to be physiologically relevant.

Our results demonstrate that chronic flumazenil and FG 7142 administration produces qualitatively similar increases in GABA-dependent chloride uptake in cultured neurons, although the effects of FG 7142 appeared to be greater than those of flumazenil. The similarity between flumazenil and FG 7142 may indicate, as suggested by behavioral studies [20], that chronic administration promotes GABA-negative effects of flumazenil. The mechanism for these alterations remains uncertain. Our studies in animals suggest that chronic flumazenil upregulates the entire $GABA_A$ receptor complex [8], so that increased chloride uptake may reflect an increase in available receptors.

Similar hypotheses can be presented to account for the effect of chronic FG 7142. It is also possible that the effects of these agents are nonspecific, although the lack of change in GABA-independent chloride uptake and the attenuation of the FG 7142 effect by flumazenil strongly argue in favor of a specific effect at the benzodiazepine site. Preliminary evidence from acute and chronic lorazepam administration suggests that benzodiazepine agonists may exert effects on $GABA_A$ receptor gene expression ([31] and Miller *et al.*, unpublished observations). Whether chronic antagonists or inverse agonists act at the genomic level or at the post-translational level will be of interest in examining $GABA_A$ receptor regulation. The present study illustrates the utility of the primary neuron culture system and chloride uptake techniques in assessing modulation of the $GABA_A$ receptor, and in examining effects of chronic benzodiazepine administration on the receptor complex.

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