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Chronic Clonazepam Administration Decreases γ-Aminobutyric Acid$_A$ Receptor Function in Cultured Cortical Neurons

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SUMMARY

Chronic benzodiazepine administration has been reported to decrease γ-aminobutyric acid$_A$ (GABA$_A$) receptor function in animals and may alter benzodiazepine binding in neuronal cultures. To assess GABA$_A$ receptor function in neuronal cultures exposed to chronic clonazepam, we measured muscimol-stimulated chloride uptake in chick cerebral cortical cultures treated acutely and for 2, 4, and 10 days. Acute clonazepam administration (1 μM) led to an increase in GABA-related chloride uptake at lower doses of muscimol. After chronic clonazepam (1 μM), maximal uptake was markedly decreased at day 10, but maximal uptake was unchanged after 2- and 4-day treatments. Benzodiazepine receptor binding was decreased by approximately 60% after 10 days due to a decrease in receptor number. Decreases in chloride uptake were also observed after 10 days of treatment with 0.1 and 10 μM clonazepam. Concomitant treatment with 0.1 μM Ro15-1788 abrogated the effect of 0.1 μM clonazepam on chloride uptake. Chronic clonazepam treatment (1 μM) did not alter total cellular protein, cellular protein synthesis or degradation, or percentage of neuronal cells, as determined morphologically and by [^{3}H]ouabain binding.

Tolerance to the effects of benzodiazepines has been reported in animals and humans and in some cases limits the use of these drugs (1, 2). Despite ample behavioral confirmation of the development of tolerance, the neurochemical mechanisms underlying tolerance remain uncertain. Because benzodiazepines exert their effects at a specific binding site located on the GABA$_A$ receptor, efforts have focused on changes at this site or other sites on the receptor after chronic drug administration. Several groups have recently reported that chronic benzodiazepine administration down-regulates benzodiazepine receptor binding and possibly GABA$_A$ receptor function as well (3–5).

Prior studies have utilized tissue from animals treated chronically and, as such, may be influenced by nutrition, stress, hormonal interactions, and a variety of other stimuli (6). Use of an *in vitro* model, in which these possible confounding factors may be avoided, allows corroboration of *in vivo* results. Prior studies of benzodiazepine binding in cultured cortical neurons have produced conflicting results (7–12). Studies have reported decreases or no change in benzodiazepine binding after chronic benzodiazepine administration. Techniques to assess function of the GABA$_A$ receptor in chloride transport in cultured cells have been developed recently (13). In the present study, we used these methods to assess function of the GABA$_A$ receptor complex in chick cortical neuron cultures chronically exposed to clonazepam.

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Materials and Methods

Embryonated White Leghorn eggs were obtained from Truslow Farms (Chestertown, MD) and were maintained in a humidified, forced-draft, incubator at 37.5°. $^{36}Cl^-$ (specific activity, 12.5 mCi/g), [^{3}H]flunitrazepam (specific activity, 72 Ci/mmol), [^{3}H]ouabain (specific activity, 15 Ci/mmol), and [^{35}S]methionine (specific activity, 110 Ci/mmol) were obtained from New England Nuclear (Boston, MA). Muscimol, pentylenetetrazol, and L-methionine were purchased from Sigma (St. Louis, MO). Clonazepam and flurazepam were generously 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 that were staged according to the series of Hamburger and Hamilton (14). The tissue was minced into 1-mm pieces and incubated in Puck's D$_1$G medium, containing 0.5% trypsin, for 30 min at 37° without stirring. The tissue was collected by centrifugation, resuspended in 5% Eagle's minimum essential medium that contained 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, and the cells were counted in a hemacytometer. Cells were placed on collagenized vinyl plastic coverslips in the presence of cytosine arabinoside (10^{-5} M) at a density of 10^5 cells/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% minimum essential medium

ABBREVIATIONS: GABA, γ-aminobutyric acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TBPS, *t*-butylbicyclophosphorothionate.

without cytosine arabinoside, containing the appropriate concentration of clonazepam.

Drug administration. Clonazepam was dissolved in ethanol and diluted with saline to less than 1% ethanol. Final concentration in culture was less than 0.001% ethanol. Control cultures were treated with vehicle alone. Cultures were treated with 1 μM clonazepam for 1 hr and 2, 4, and 10 days. Two-day treatment was begun on day 8 of culture and 4-day treatment on day 6 of culture. Some cultures were treated with 0.1 and 10 μM clonazepam for 10 days.

$^{36}Cl^-$ uptake. Uptake of labeled chloride was performed using a modification of the method of Thampy and Barnes (13). 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 four times in 500 ml of fresh HEPES-buffered saline at 22° for 5 sec, drained, and transferred to high potassium HEPES-buffered saline (96 mM NaCl, 50 mM KCl, 1.4 mM $MgCl_2$, 1.2 mM $CaCl_2$, 1 mM NaH_2PO_5, 20 mM HEPES, pH 7.4) containing $^{36}Cl^-$ (5–10 μCi/ml) and muscimol (1–100 μM). In some experiments, 6 μM picrotoxin was added concurrently with muscimol. After intervals ranging from 10 sec to 30 min, uptake was terminated by transfer of coverslips to 600 ml of an ice-cold stop solution (13), containing 50 μM pentylenetetrazol, 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 counting. Protein concentrations were determined by the method of Simpson and Sonne (15).

Morphological analysis. To assess neuronal survival in clonazepam- and vehicle-treated cultures, cells were plated at one-half density and maintained as above. After 10 days, 10 high-power fields from treated and control cultures were examined and nonneuronal cells were counted.

Protein synthesis and degradation. For cellular protein synthesis, cultures were incubated with 20 nM [^{35}S]methionine (30 min at 37°). Cultures were then washed to remove unincorporated radioactivity. Sodium dodecyl sulfate/Nonidet P-40/urea (0.2%/2%/8 M) was added, and aliquots were removed. Trichloroacetic acid 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 counting. For cellular protein degradation, cultures were labeled with 2 nM [^{35}S]methionine (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 trichloroacetic acid was added to a final concentration of 10%. Samples were incubated at 4° for 30 min, followed by centrifugation (15,000 × *g*), and supernatants were counted by scintillation counting.

[^{3}H]Ouabain binding. Binding was performed as described by Hauger *et al.* (16). Binding appears to be highly specific for neurons (16).[1] Cells were scraped from dishes and washed with 50 mM Tris-HCl (pH 7.4 at 4°) at 1,000 × *g* for 10 min at 4°. Cells were homogenized by hand (five 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 gently resuspended in 100 mM Tris·HCl, 10 mM $MgCl_2$, 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 buffer and counted by scintillation counting.

Benzodiazepine binding. To remove residual clonazepam or vehicle, membranes were prepared as described above. After washing, membranes were suspended in 10 mM phenylmethylsulfonyl fluoride, 10 mM EDTA (0.25 ml/dish), and dialyzed, using 15,000 molecular weight cutoff tubing, for 48–72 hr at 4° in 4 liters of 25 mM potassium phosphate buffer (pH 7.4). Dialysate was changed twice (total dialysis volume, 12 liters). 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 (0.1–5 nM). An additional set of samples were 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 counting. Data analysis was performed using the EBDA programs (17).

Data analysis. In chloride uptake experiments, non-GABA-related uptake was subtracted from total uptake to yield GABA-related uptake. GABA-independent uptake was not altered by clonazepam treatment and varied by 10–20% among assays. 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

Effects of chronic clonazepam on cultured neurons. Chronic clonazepam might alter $GABA_A$ receptor function indirectly, through effects on total cellular protein, protein synthesis and degradation, percentage of nonneuronal cells present, or cell internal volume.

Total cellular protein. Protein determinations in coverslips treated with clonazepam (1 μM for 10 days) were similar to vehicle treatment in a large number of experiments. Results for three representative experiments are presented in Table 1.

Protein synthesis and degradation. Total cellular protein synthesis in clonazepam-treated cells (1 μM for 10 days) was similar to protein synthesis in vehicle-treated cells (Fig. 1). Similarly, no change in cellular protein degradation was observed in clonazepam-treated compared with vehicle-treated cultures.

Percentage of nonneuronal cells in culture. The presence of nonneuronal cells in culture was determined both by morphological analysis and by ouabain binding. In cultures treated with clonazepam (1 μM for 10 days), the number of nonneuronal cells in 10 representative fields was vehicle, 16.4 ± 0.8; clonazepam, 16.2 ± 1.1 (mean ± SE). Both vehicle- and clonazepam-treated cultures contained greater than 95% neuronal-appearing cells after 10 days of culture. Ouabain binding was similar in membranes prepared from vehicle- and clonazepam-treated cultures (vehicle, 1.98 ± 0.09 pmol/mg of protein; clonazepam, 1.94 ± 0.10 pmol/mg of protein; mean ± SE; $n = 6$ in each group from a representative experiment repeated twice). Internal volume, determined as complete equilibration of chloride (30 min) in the absence of muscimol was similar: vehicle, 309 ± 31 nmol/mg of protein; clonazepam, 334 ± 18 nmol/mg of protein (mean ± SE; $n = 3$). These results indicate

TABLE 1

Effects of chronic clonazepam on total cellular protein

Results are from three representative experiments, mean ± standard error, 24 determinations in each group in each experiment. There are no significant differences.

	Protein	
	Vehicle	Clonazepam
	μg/coverslip	
Expt. 1	1860 ± 203	1798 ± 156
Expt. 2	2673 ± 522	2664 ± 343
Expt. 3	1861 ± 258	2195 ± 323

[1] D. Brenneman, personal communication.

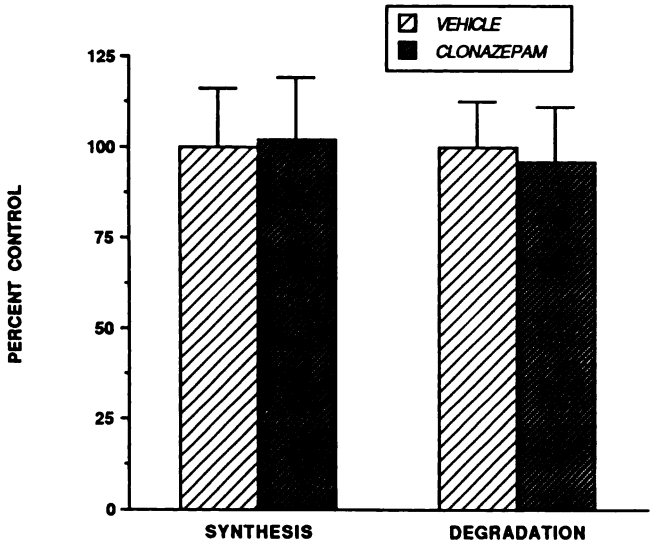

Fig. 1. Effects of chronic clonazepam administration on cellular protein synthesis and degradation. Protein synthesis was determined after a 30-min incubation with 25 μM [^{35}S]methionine in cultures treated with 1 μM clonazepam or vehicle for 10 days. Results are mean ± standard error for each group. Protein degradation was determined in similarly treated cultures 6 hr after a 24-hr incubation with [^{35}S]methionine (2 nM). Results are mean ± standard error; $n = 6$ for each group. There are no significant differences.

TABLE 2

Effects of chronic clonazepam administration on benzodiazepine binding

Cultures were treated with 1 μM clonazepam or vehicle for 10 days. Membranes were extensively dialyzed, followed by Rosenthal-Scatchard analysis using [^{3}H] flunitrezepam. Results are mean ± standard error of three experiments, with two separate analyses per experiment.

	K_d	B_{max}
	nM	*pmol/mg of protein*
Vehicle	2.12 ± 0.17	1.72 ± 0.15
Clonazepam	1.93 ± 0.33	1.08 ± 0.22[a]

[a] $p < 0.05$ compared with vehicle.

little effect of chronic clonazepam on total cellular protein, cellular protein synthesis and degradation, presence of nonneuronal cells, and cell internal volume.

Benzodiazepine binding. To assess benzodiazepine binding after acute and chronic clonazepam treatment, membranes from cultures treated for 10 days with clonazepam (1 μM) or vehicle were prepared and extensively dialyzed to remove residual clonazepam or vehicle. Rosenthal-Scatchard analyses were then performed using [^{3}H]flunitrazepam (Table 2). After chronic treatment, there was no change in K_d but B_{max} was substantially reduced compared with vehicle-treated controls. In three experiments in which two Rosenthal-Scatchard analyses were performed in each case, the mean decrease in B_{max} was 60%.

Chloride uptake interval. To ensure that the 10-sec uptake interval represents initial uptake rather than equilibrium of chloride within an intracellular compartment, muscimol-stimulated and unstimulated uptake were determined at intervals from 10 sec to 10 min (Fig. 2). Uptake increased rapidly over the initial 1-min period in both muscimol-treated and control cells and then increased gradually up to 10 min. Chloride uptake in the first 30 sec was approximately linear in both stimulated and unstimulated cells (Fig. 2, *inset*) and uptake at

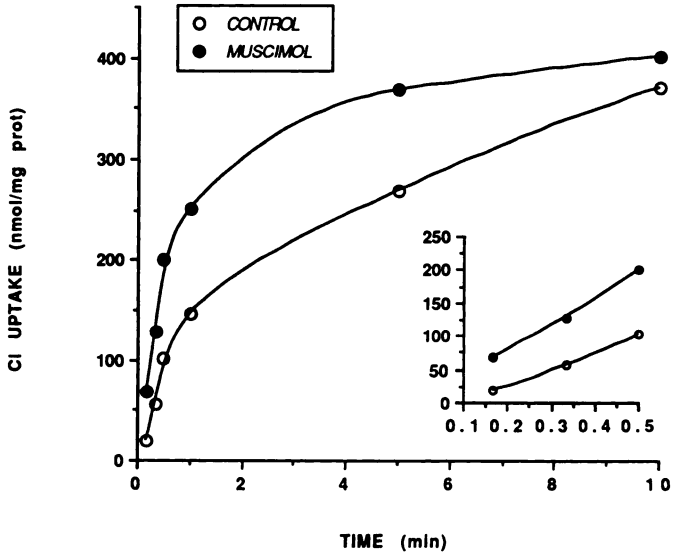

Fig. 2. Time course of muscimol-dependent and -independent chloride uptake in cultured neurons. Muscimol concentration was 50 μM, and control cultures were treated with vehicle only. Uptake was terminated as described after intervals ranging from 10 sec to 10 min. Results are means of two determinations from a representative experiment repeated twice. *Inset* shows uptake in control- and muscimol-treated cultures from 10 to 30 sec.

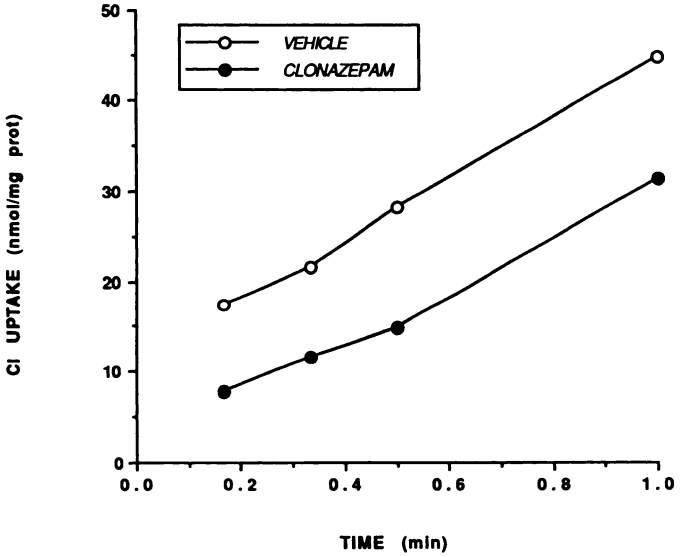

Fig. 3. Time course of muscimol-dependent chloride uptake in cultured neurons treated with chronic clonazepam. Cultures were treated with 1 μM clonazepam or vehicle for 10 days. Muscimol concentration was 10 μM, and uptake intervals varied from 10 to 60 sec. Results are means of three or four determinations. Uptake remained linear for both treatments from 10 to 60 sec ($r > 95$ for both), and ratios between vehicle and clonazepam uptake remained constant ($p < 0.05$).

10 sec was less than 15% of uptake at 10 min in both groups. Results are similar to those obtained by Thampy and Barnes (13) and indicate the validity of the 10-sec incubation interval. Chronic clonazepam administration (1 μM for 10 days) did not alter uptake characteristics in the first minute of incubation (Fig. 3).

Effects of picrotoxinin. Because chloride uptake mediated by the $GABA_A$ receptor is sensitive to chloride channel antagonists, the effect of the antagonist picrotoxin was determined on muscimol-dependent and -independent chloride uptake in

cultured neurons. Picrotoxin (6 μM) markedly reduced muscimol-stimulated uptake in cultured neurons (Fig. 4). Results were similar in neurons treated with clonazepam for 10 days (data not shown). With regard to muscimol-independent uptake, picrotoxin had no effect on chloride uptake in either vehicle-treated or clonazepam-treated (1 μM for 10 days) neurons (vehicle, 55 ± 3 nmol/mg of protein; clonazepam, 56 ± 4 nmol/mg of protein; mean ± SE; $n = 3$ for each). These data confirm the specificity of uptake analysis for the $GABA_A$ receptor-gated chloride uptake. In addition, the lack of picrotoxin effect on muscimol-independent chloride uptake indicates the lack of a significant contribution of endogenous GABA to chloride uptake in either treated or control cultures.

Acute clonazepam administration. To assess the effects of acute clonazepam administration on muscimol-stimulated chloride uptake, untreated cultures were incubated with 1 μM clonazepam for 1 hr before chloride uptake determinations (Fig. 5). Uptake was significantly increased at lower muscimol concentration evaluated in clonazepam-treated compared with vehicle-treated culture, but maximal uptake was not increased with clonazepam treatment.

Chronic clonazepam administration. Treatment of cultures with 1 μM clonazepam for 2 and 4 days did not alter maximal muscimol-dependent chloride uptake, compared with vehicle-treated cultures (Fig. 6). After 10 days of treatment, muscimol-dependent chloride uptake was markedly decreased, compared with vehicle-treated cultures (Fig. 7). Uptake was also decreased at muscimol concentrations of 25 and 100 μM after 10 days, compared with cultures treated with clonazepam for 2 and 4 days. There was a tendency toward decreased uptake at a muscimol concentration of 2 μM, but this did not achieve significance ($p < 0.15$). Clonazepam treatment for any interval did not alter muscimol-independent chloride uptake (data not shown).

To assess the dose dependency of clonazepam effects on muscimol-stimulated chloride uptake, cultures were treated with 0.1 and 10 μM clonazepam, in addition to 1 μM, as described above, for 10 days (Fig. 8). At 10 days, uptake at 0.1, 1, and 10 μM clonazepam concentrations was decreased, compared with vehicle, and uptake at 1 and 10 μM was significantly decreased, compared with 0.1 μM and vehicle. Uptake was similar at 1 and 10 μM.

To address the specificity of the effect of clonazepam on $GABA_A$ receptor function, cultures were treated simultaneously with 0.1 μM Ro15-1788 (flumazenil) and 0.1 μM clonazepam for 10 days or with Ro15-1788 alone. Treatment with this concentration of Ro15-1788 did not affect chloride uptake, although

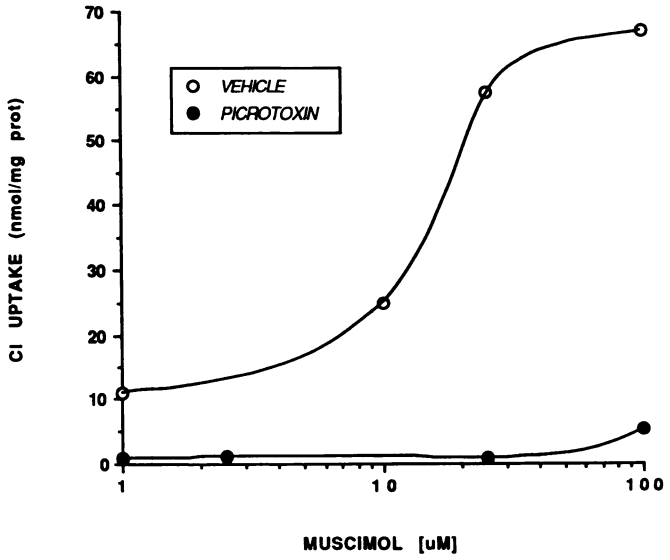

Fig. 4. Effects of picrotoxin on muscimol-dependent chloride uptake in cultured neurons. Uptake was performed in 10-day cultures over a 10-sec interval in the presence or absence of 6 μM picrotoxin. Results are means of two or three determinations from a representative experiment repeated three times.

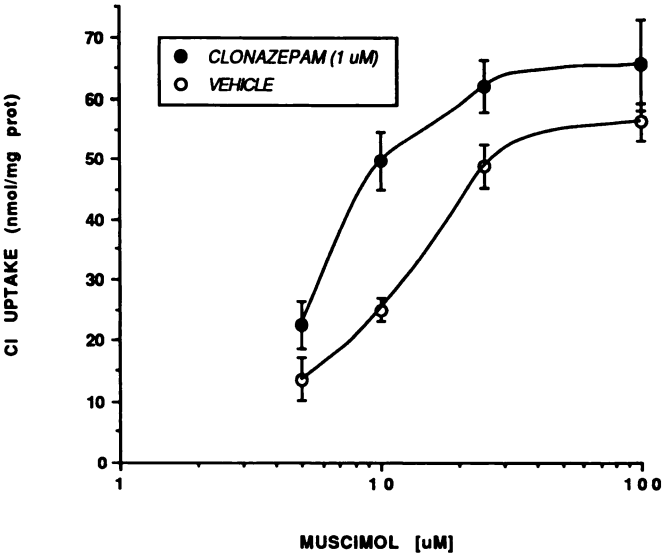

Fig. 5. Effects of acute clonazepam on muscimol-dependent chloride uptake in cultured neurons. Ten-day cultures were treated with 1 μM clonazepam or vehicle for 1 hr before uptake determinations. Uptake interval was 10 sec. Muscimol-independent chloride uptake was unchanged after clonazepam treatment. Results are means of four to six determinations from two separate experiments. Differences are significant ($p < 0.05$) except at 100 μM muscimol.

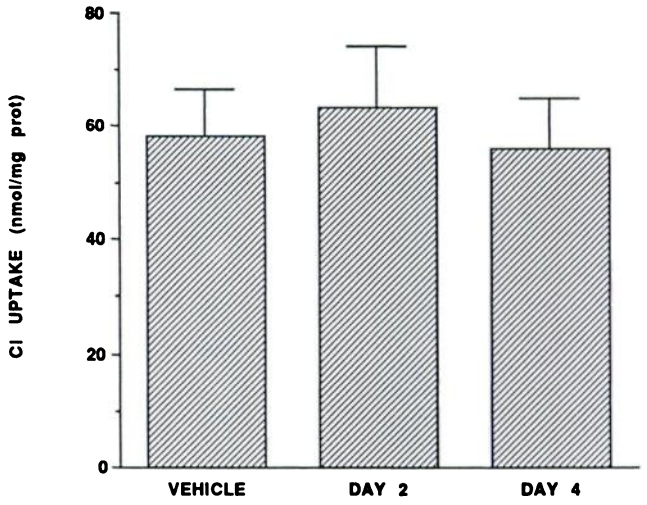

Fig. 6. Effects of chronic clonazepam administration for 2 and 4 days on maximal muscimol-dependent chloride uptake. Cultures were treated with 1 μM clonazepam for the specified intervals, ending at 10 days of culture. Vehicle treatment was administered for 4 days. Uptake interval was 10 sec. Muscimol-independent chloride uptake was unchanged after any clonazepam treatment interval. Muscimol concentration was 100 μM. Results are mean ± standard error of four to six determinations from a representative experiment repeated three times. Differences are not significant.

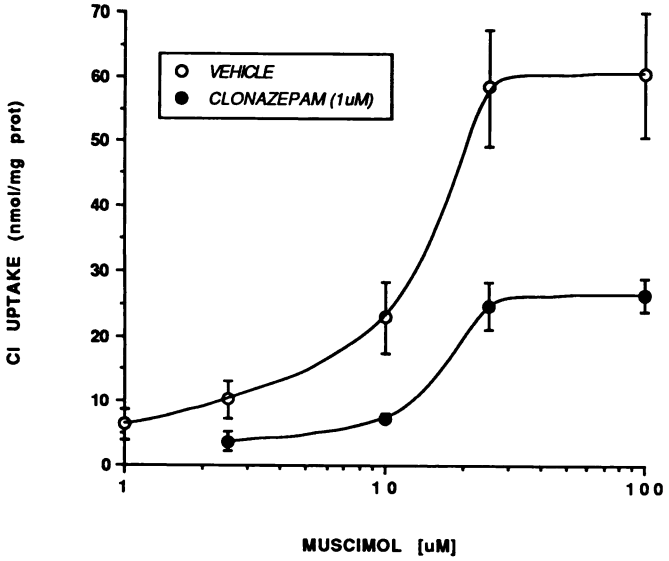

Fig. 7. Effects of chronic clonazepam administration for 10 days on muscimol-dependent chloride uptake. Cultures were treated with 1 μM clonazepam or vehicle for 10 days and analyses were performed at day 10 of culture. Uptake interval was 10 sec. Muscimol-independent chloride uptake was unchanged after clonazepam treatment. Muscimol concentrations were 1–100 μM. Results are mean ± standard error of three separate experiments performed in triplicate or quadruplicate. Differences are significant ($p < 0.05$) at muscimol concentrations of 10, 25, and 100 μM.

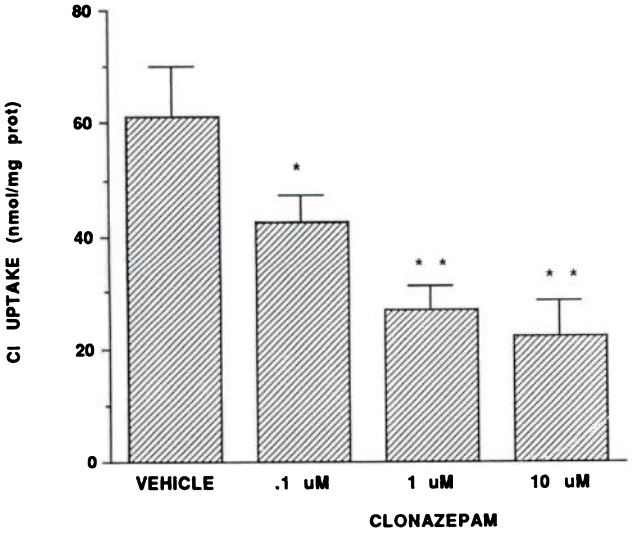

Fig. 8. Dose effect of chronic clonazepam administration for 10 days on maximal muscimol-dependent chloride uptake. Cultures were treated with 0.1, 1, or 10 μM clonazepam for 10 days and analyses were performed at day 10 of culture. Vehicle-treated cultures received vehicle from the highest clonazepam dose. Uptake interval was 10 sec. Muscimol-independent chloride uptake was unchanged compared with vehicle after any clonazepam treatment. Muscimol concentration was 100 μM. Results are mean ± standard error of four to six determinations in a representative experiment repeated twice. *$p < 0.05$ versus vehicle; **$p < 0.05$ versus vehicle and 0.1 μM clonazepam.

uptake was altered with higher concentrations.[2] In concomitantly treated cultures, uptake was similar to Ro15-1788 alone or vehicle at each muscimol concentration (Fig. 9), indicating abrogation of the clonazepam effect. Antagonism of clonazepam effect by the specific benzodiazepine site antagonist Ro15-1788

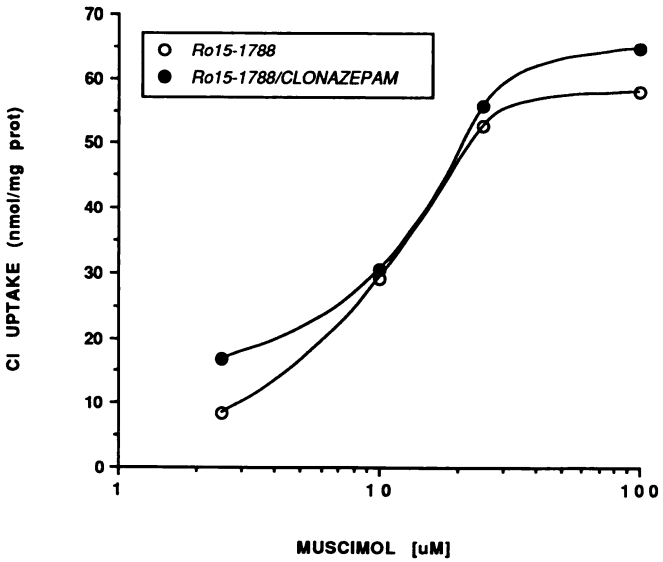

Fig. 9. Effects of concomitant treatment with Ro15-1788 on muscimol-dependent chloride uptake after chronic clonazepam administration. Cultures were treated with 0.1 μM Ro15-1788 alone or in combination with 0.1 μM clonazepam for 10 days and analyses were performed at day 10 of culture. Uptake interval was 10 sec. Muscimol-independent chloride uptake was unchanged compared with vehicle after either treatment. Muscimol concentrations were 2.5–100 μM. Results are mean ± standard error of three or four determinations in a representative experiment repeated twice. Differences are not significant at any muscimol concentration.

indicates that clonazepam acts through the benzodiazepine site and supports the specificity of the effect of clonazepam on GABA-dependent chloride uptake.

Discussion

Prior studies indicate that muscimol, flunitrazepam, and TBPS binding are present in chick embryos, and binding at each site increases during embryonic development (18–21). Studies in cultured chick cortical neurons show analogous increases in binding up to 10 days in culture (22). Similar results were obtained in our studies.[3] These data indicate that the cultured neuron system as used in the present study may serve as a model for the development of the $GABA_A$ receptor complex (23). The duration of culture in the present study, 10 days, was chosen based on increases in binding sites up to this point.

The methodology for assessing GABA-related chloride uptake appears to be sensitive and pharmacologically specific. Both muscimol-dependent and -independent uptake increased with incubation time, and the 10-sec interval used in this study represents a fraction of total uptake. In addition, muscimol-dependent uptake was blocked by the chloride channel antagonist picrotoxin, whereas this compound had no effect on muscimol-independent uptake. Results obtained in this study were similar to those previously reported by Thampy and Barnes (13) using a similar culture system and chloride uptake techniques. With regard to sensitivity of the assay, results of separate experiments show variation of 15–20% between determinations. Although it is unlikely that a small effect (<25%) could be demonstrated by a limited number of experiments, the

[2] L. G. Miller *et al.*, unpublished data.

[3] L. G. Miller and C. L. Weill, unpublished data.

sensitivity is adequate for effects of the magnitude (40–70%) observed with chronic clonazepam.

Our results indicate that chronic clonazepam administration for 10 days leads to decreases in $GABA_A$ receptor function. The magnitude of the change in maximal muscimol-stimulated chloride uptake is approximately 50–60% overall and as great as 70% in some experiments. Similar decrements were observed at lower doses of muscimol, although the differences were not statistically significant at the lowest doses of muscimol evaluated, in part due to assay variance. If clonazepam administration decreases $GABA_A$ receptors, it is also possible that sufficient numbers of GABA-coupled receptors exist after chronic clonazepam treatment to provide similar chloride uptake with lower muscimol concentrations. Results in tissue preparations also indicate a lack of statistical significance at low muscimol concentrations after chronic benzodiazepine administration (3).

Several lines of evidence support a specific effect of clonazepam as the cause of the observed decrease in GABA-related chloride uptake. First, no change was observed after clonazepam treatment on total cellular protein or on cellular protein synthesis or degradation. These data argue against a nonspecific effect on protein synthetic mechanisms. Second, no alteration in the percentage of neuronal or nonneuronal cells was observed by morphological analysis or by ouabain binding. The latter technique appears to be highly specific for neurons (16).[1] It is, therefore, unlikely that a decrease in neuronal cells accounts for the observed alterations. In addition, internal volume as measured by equilibrium uptake of chloride was unchanged after chronic clonazepam, indicating no significant change in membrane stability.

Third, experiments involving simultaneous administration of clonazepam and the specific benzodiazepine antagonist Ro15-1788 (flumazenil) demonstrated blockade of the clonazepam effect by Ro15-1788. These experiments were performed with low concentrations of clonazepam and Ro15-1788, because concentrations of Ro15-1788 of 1 μM or greater alter chloride uptake.[4] Ro15-1788 alone did not alter uptake compared with vehicle, but in combination with clonazepam no decrement in uptake was observed. These data suggest that the effect of clonazepam is mediated at the benzodiazepine site and is, thus, benzodiazepine specific rather than involving an unrelated action of clonazepam.

Prior studies have not addressed the effects of chronic benzodiazepine administration on $GABA_A$-related chloride transport, although several studies assessed effects on binding. Shibla *et al.* (9) reported no change in benzodiazepine binding in mouse-derived cultured treated for 3 weeks with 1 μM diazepam. Similar results were reported by Farb *et al.* (11) in a chick system and by Maloteaux *et al.* (10) in rat-derived cultures. In contrast, Sher *et al.* (7) reported decreased binding in mouse spinal cord cultures exposed to diazepam for 1 week and decreased electrophysiological potentiation of GABA responses by diazepam. Subsequently, Sher and Machen (8) found similar decreases in binding in mouse-derived cortical cell cultures after exposure to 200 nM clonazepam for 14 days. Recently, Roca *et al.* (12) reported no change in binding in chick cortical cultures after chronic flurazepam (10 μM) but a decrease in coupling of the benzodiazepine and GABA sites. The wide variations in results of these studies may be due in part to species differences, drug differences, and variations in binding techniques.

Similarly, conflicting results have been reported in studies addressing the effects of chronic benzodiazepine administration in intact animals. Prior reports have indicated increases (24), decreases (25), and no change (26, 27) in benzodiazepine binding in animals treated with various benzodiazepines under various protocols. Several more recent studies have reported decreases in benzodiazepine binding after chronic benzodiazepine administration (4, 6). With regard to $GABA_A$ receptor function, one study reported a decrease in iontophoretic sensitivity to GABA in diazepam-treated animals (28). Two studies from our laboratory (6, 29) and the report of Marley and Gallager (5) indicated decreased GABA-dependent chloride uptake in rodents treated with chronic benzodiazepines. In addition, we found a similar decrease in GABA-related chloride uptake in intact embryos treated with lorazepam for intervals similar to those used in this study (30).

Thus, although the literature is conflicting, studies in several systems reported decreased $GABA_A$ receptor coupling or function associated with chronic benzodiazepine exposure. The results of the present study are consistent with these reports. The magnitude of the decrease in maximal GABA-related uptake (50–60%) is similar to that reported in cortical tissue from treated animals. In addition, the time course of changes in uptake is similar to that reported in intact chick embryos (30). That these results may have physiological significance is also supported by the similarity of the lower clonazepam concentrations (0.1 μM) and the concentrations expected in brain during clinical use of the drug, based on extrapolations from plasma concentrations and brain uptake of other benzodiazepines (31, 32).

The mechanism of action of clonazepam in leading to decreased GABA-related chloride uptake remains uncertain. Results from binding studies, in accord with some but not all prior studies, indicate that a decrease in benzodiazepine binding can be accounted for by a decrease in number of sites. It is possible that the entire $GABA_A$ receptor complex is down-regulated after chronic benzodiazepine administration, with concomitant decreases in chloride uptake. Alternatively, reductions in benzodiazepine binding and receptor function might reflect alterations in coupling of the subunits that comprise the receptor complex. Studies based on gene expression of the $GABA_A$ receptor subunits will be useful in addressing this issue.

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