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Benzodiazepine Interactions with $GABA_A$ Receptors on Chick Ciliary Ganglion Neurons

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SUMMARY

γ-Aminobutyric $acid_A$ ($GABA_A$) receptors on chick ciliary ganglion neurons can be modulated by benzodiazepines and identified by radiolabeled benzodiazepine binding. Enhancement of submaximal GABA responses by benzodiazepines was demonstrated using a multibarrel pipette to construct complete benzodiazepine dose-response curves for single cells in culture. EC_{50} values of 22 ± 5 nM, 1.1 ± 0.3 μM, and 4.6 ± 0.5 μM were obtained for flunitrazepam, clonazepam, and chlordiazepoxide, respectively. Chlordiazepoxide shifted the GABA dose-response curve to lower GABA concentrations without increasing the maximal response to GABA, demonstrating that benzodiazepines enhance the GABA response by increasing the receptor affinity for GABA. The imidazodiazepine Ro15-1788 potentiated the GABA response with an EC_{50} of 250 ± 70 nM, and Ro5-4864 (chlorodiazepam) partially blocked the GABA response both in the presence and absence of chlordiazepoxide. Scatchard analysis of data from binding studies with [^{3}H]flunitrazepam to ganglion membrane homogenates was consistent with the presence of a single class of high affinity sites with a K_D of 34 ± 6 nM and a B_{max} of 145 ± 26 fmol/mg of protein. Several lines of evidence indicated that the sites were associated with $GABA_A$ receptors. The K_D of [^{3}H]flunitrazepam binding was similar to the EC_{50} for flunitrazepam modulation of the GABA response. The level of [^{3}H]flunitrazepam binding was enhanced approximately 50% over control levels by GABA. The binding was decreased both by clonazepam and by Ro5-4864 at concentrations similar to those required for the compounds to modulate the GABA response. These studies demonstrate that ciliary ganglion $GABA_A$ receptors are similar in major respects to $GABA_A$ receptors in the central nervous system but may differ in minor pharmacological properties.

GABA is the primary neurotransmitter mediating inhibitory synaptic transmission in the vertebrate CNS. The inhibition is brought about primarily by activation of $GABA_A$ receptors and their associated chloride channels in the neuronal plasma membrane. BDZ agonists allosterically enhance submaximal GABA responses (1) and have been used in radioactive form to identify $GABA_A$ receptors in the CNS (2, 3). BDZ and GABA binding sites on the receptor may be associated with separate subunits, designed as α and β, respectively (4), and recently, cDNAs encoding the α and β subunits of the receptor have been isolated and used to express a functional $GABA_A$ receptor in *Xenopus* oocytes (5).

$GABA_A$ receptors have also been identified electrophysiologically on neurons in the periphery (6) and appear to be modulated by BDZs as in the CNS (1). Although radiolabeled BDZs have been used to identify specific binding sites in a number of nonneuronal peripheral tissues, the sites are not thought to represent neurotransmitter receptors and instead appear to be associated with mitochondrial membranes (7, 8). An exception to this occurs in bovine adrenal glands in which modulation of $GABA_A$ receptors by BDZs has been described and specific binding of radiolabeled BDZs to the receptor has also been reported (9, 10). The relationship of the $GABA_A$ receptor on peripheral neurons to the CNS receptor remains unclear. In most instances no physiological role has been identified for the receptor on peripheral neurons. Only in the enteric nervous system is there strong evidence for GABA-mediated synaptic transmission (11).

Neurons in the chick ciliary ganglion have classical $GABA_A$ receptors that activate a chloride conductance, respond to GABA and muscimol as agonists, and recognize bicuculline and picrotoxin as antagonists (12). The receptors are of potential physiological significance for ganglionic function because application of micromolar concentrations of GABA completely blocks transmission through the ganglion (12). No endogenous source of GABA has yet been identified for the receptors.

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ABBREVIATIONS: GABA, γ-aminobutyric acid; BDZ, benzodiazepine; CDPX, chlordiazepoxide; CNS, central nervous system; DMSO, dimethyl sulfoxide; [^{3}H]FNZM, [^{3}H]flunitrazepam; HEPES, *N*-2-hydroxyethyl-piperazine-*N*′-2-ethanesulfonic acid; n_H, Hill coefficient; PMSF, phenylmethylsulfonyl fluoride; Ro15-1788, ethyl-8-fluoro-5,6-dihydro-5-methyl-6-oxo-4 *H*-imidazo[1,5-a][1,4]benzodiazepine-3-carboxylate; Ro5-4864, chlorodiazepam.

We show here that BDZs modulate $GABA_A$ receptors on chick ciliary ganglion neurons as they do $GABA_A$ receptors in the CNS. In addition, we use [^{3}H]FNZM, a radiolabeled BDZ, to detect a class of binding sites in ganglion homogenates that have the properties expected for $GABA_A$ receptors as predicted from the electrophysiological studies. In most respects the ganglionic receptor appears similar to the CNS receptor, although the data suggest that certain pharmacological differences exist. Some of these results have been described in a preliminary account (13).

Materials and Methods

Cell cultures. Ciliary ganglion neurons dissociated from 8-day chick embryos were grown in cell culture on a substratum of collagen and lysed fibroblasts as previously described (14). Cultures contained 1–2 $\times 10^4$ neurons in 35-mm plastic dishes and were maintained in medium consisting of Eagle's minimal essential medium with 3% (v/v) embryonic chick eye extract, 10% (v/v) heat-inactivated horse serum, 50 units/ml penicillin, and 50 μg/ml streptomycin. Spinal cords were dissociated from 7-day chick embryos and grown in cell culture at a density of 3 $\times 10^5$ cells per 35-mm dish as previously described (15) except the cultures were not exposed to cytosine arabinoside and were maintained in medium containing 5% (v/v) embryonic chick extract.

Electrophysiology. Intracellular recordings were obtained from ciliary ganglion neurons after 4–7 days in culture and from spinal cord neurons 8–15 days in culture using techniques previously described (12, 16). Cultures were maintained at 37° on the stage of an inverted microscope and continuously perfused with a medium composed of 137 mM NaCl, 5.4 mM KCl, 0.81 mM $MgSO_4$, 0.92 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 5.4 mM $CaCl_2$, 5.6 mM glucose, 2.5 mM HEPES (pH 7.4), and the essential amino acids and vitamins present in Eagle's minimal essential medium.

Neurotransmitter sensitivities were determined by measuring changes in membrane conductance caused by the transmitter being pressure ejected (1–3 psi for 1–3 sec) from single barrel pipettes (4–6 μm tip diameters) positioned 10–20 μm from the soma (12, 17). In most experiments multibarrel pipettes were also used (see below). Experiments with K^+ and dye-filled micropipettes indicated that the procedure completely immerses the soma in the ejected solution (17, 18). Transmitter sensitivities determined in this way are probably the "summed" response of receptors on the soma. Input resistances were measured using a single intracellular electrode in conjunction with an amplifier and a Wheatstone bridge circuit (model M701; WPI, Inc., New Haven, CT); constant current pulses of 0.05–0.15 nA amplitude and 50 msec duration were injected, and the resulting membrane hyperpolarizations were recorded before and during transmitter exposure. The transmitter-induced conductance was then calculated as the difference between the maximum conductance measured in the presence of transmitter and the resting conductance prior to application of transmitter. Data were accepted only when the neuron had a resting potential more negative than −45 mV and could fire overshooting action potentials in response to intracellular stimulation and when the recording electrode was no more than 3 mV out of balance when withdrawn from the neuron. Most cells had resting potentials of −50 to −65 mV. GABA was applied at 10 μM to elicit a response less than half maximal when testing the effects of potentiating drugs; higher concentrations of GABA were used when inhibitory drugs were tested.

In most cases complete dose-response curves were performed on single neurons using a seven-barrel puffer pipette (19) fabricated from multibarrel glass capillary tubing (WPI, Inc.). Individual barrels having tips of 7-8 μm were filled with recording solution containing the desired drug. A one-half inch long bent hypodermic tubing (HTX-25; Small Parts, Inc., Miami, FL) was then inserted into each barrel and fastened with epoxy. Silastic tubing was used to connect each pipette to an eight-spigot valve assembly (Hamilton, Reno, NV). To prevent a contribution to the measured response by a possible continuous slow leak of drugs from the pipette barrels, the multibarrel pipette was held well above the cell between tests and only positioned within 50 μm at the start of a drug trial. The continuous perfusion system prevented a build-up of drugs in the culture bath.

When testing the effects of compounds on the GABA response, the compound was applied for 10–15 sec, followed within 1 sec by application of GABA. This arrangement was chosen to ensure a steady state of binding for the compound while avoiding simultaneous application of the compound and GABA that would have diluted the concentration of GABA reaching the cell. Confirmation that the procedure achieved steady state binding comes from the observations that increasing either the time of drug application or the interval between drug and GABA application up to 2 sec did not alter the effect of the drug on the GABA response. To compensate for small time-dependent changes in the basal GABA response, measurements were made throughout the time course of an experiment (usually 20 min) and linear regression analysis of the data was used to estimate the basal response at times corresponding to individual drug tests.

The per cent change in the GABA response (α) was calculated by the equation:

$$\alpha = \left[\frac{g_{GABA'}}{g_{GABA}} - 1\right] \times 100$$

where g_{GABA} is the basal GABA response obtained as described above and $g_{GABA'}$ is the GABA response determined within 1 sec after application of the drug. For each neuron, the concentration giving the half-maximal drug effect (EC_{50}) and the maximal effect (α_{max}) were estimated by Eadie-Hofstee analysis. For some dose-response curves, best theoretical one-site computer fits to the data were determined by nonlinear least squares analysis.

All BDZs and related compounds were dissolved in DMSO and used at a final DMSO concentration of ≤0.1%. Control experiments showed that 0.1% DMSO had no effect on the GABA response (0.3% had variable effects).

[^{3}H]FNZM Binding. [^{3}H]FNZM binding was examined in membrane homogenates from ciliary ganglia of 16–19-day embryonic chicks. Comparable studies on cell cultures were prevented by the limited amounts of material available. No differences have been found in [^{3}H]FNZM binding to $GABA_A$ receptors in membrane fractions of embryonic chick brain and membrane homogenates of brain neurons in culture (20), encouraging the present comparison of binding studies in ganglion membrane homogenates with electrophysiological studies on neurons in cell culture. The dissected ganglia were cleaned of processes in a Ca^{2+}, Mg^{2+}-free salt solution (123 mM NaCl, 5.4 mM KCl, 11 mM $NaPO_4$, pH 7.4, with 0.1% glucose), and stored frozen at −70° until use. To prepare the membrane homogenates, frozen ganglia were thawed and immediately homogenized in a 10-fold excess (v/v) of solution containing 200 mM sucrose, 1 mM EDTA, 10 mM HEPES (pH 7.4), and a mixture of protease inhibitors consisting of 0.5 mg/liter leupeptin, 0.7 mg/liter pepstatin A, and 0.2 mM PMSF. The homogenate was centrifuged at 20,800 $\times g$ for 50 min at 4° to collect particulate debris, and the pellet was resuspended in 10 mM HEPES (pH 7.4), containing the protease inhibitors listed above, and assayed for [^{3}H]FNZM binding sites in aliquots containing 9–10 ciliary ganglion equivalents (0.1–0.15 mg of protein) in 100-μl reaction volumes. Rinsing of the pellet was avoided to reduce loss of material. Binding reactions were carried out in the dark in a balanced salt solution modified from that of Dichter and Fischbach (21) to contain 5.0 mM HEPES (pH 7.4) instead of Tris·HCl and supplemented with leupeptin (0.65 mg/liter), pepstatin A (0.9 mg/liter), and PMSF (0.06 mM). Binding was initiated by adding [^{3}H]FNZM and was terminated by adding, per reaction vessel, 1 ml of ice-cold rinse buffer consisting only of the balanced salt solution described above except that the $CaCl_2$ concentration was 5.4 instead of 1.8 mM and the protease inhibitors were omitted. Diluted incubation mixtures were immediately poured over wet Whatman GF/B glass filters under vacuum using a single filter holder (Hoefer Scientific Instruments, San Francisco, CA). The tubes were then rinsed

twice with 1-ml aliquots of rinse buffer and the filters were washed twice (10 ml each) with the same buffer. Pilot experiments indicated that the time required for the wash procedure (approximately 30 sec) permitted no more than 10% of the specifically bound ligand to dissociate. Radioactivity retained by the filters was determined by scintillation counting with Aquasol-2 in a Beckman LS-100C counter at a counting efficiency of about 36%. Nonspecific binding was determined by including 100 μM flurazepam, a water-soluble BDZ, in the binding reaction and represented 50–60% of the total binding in most experiments. Flurazepam at 1 mM yielded the same values for nonspecific binding (two experiments). Determinations were usually performed in duplicate; some were done in triplicate. Control experiments showed that the vehicle DMSO had no effect on specific [^{3}H]FNZM binding when present at ≤0.3%. Protein was determined by the method of Lowry *et al.* (22), using bovine serum albumin as a standard. Quantitative results are expressed as the mean ± standard error for the number of determinations indicated in parentheses, with each determination being done in duplicate unless otherwise indicated.

Culture media components were obtained from GIBCO (Grand Island, NY). GABA, (+)-bicuculline, HEPES, DMSO, Folin and Ciocalteu's phenol reagent, bovine serum albumin, pepstatin A, leupeptin, and PMSF were obtained from Sigma Chemical Co. (St. Louis, MO), collagenase was obtained from Boehringer Mannheim (Indianapolis, IN), and EDTA was obtained from Mallinckrodt (St. Louis, MO). [^{3}H]FNZM (79.6, 83.9 Ci/mmol) was purchased from New England Nuclear (Boston, MA). Unlabeled BDZs were kindly provided by Dr. Peter Sorter (Hoffmann-LaRoche Inc., Nutley, NJ).

Results

Benzodiazepine modulation. The BDZs FNZM, CDPX, and clonazepam were chosen for initial testing because each has been shown to enhance GABA responses of CNS neurons in avian and mammalian species (1). Each BDZ enhanced the response of chick ciliary ganglion neurons in culture to submaximal doses of GABA (Fig. 1). The imidazodiazepine Ro15-1788 was chosen for testing because it acts as a partial agonist at the BDZ site on $GABA_A$ receptors on chick CNS neurons (19). Ro15-1788 also enhanced the GABA response of ciliary ganglion neurons (Fig. 1B). In all cases the effects were dose dependent and reversible. EC_{50} and maximal percent enhancement (α_{max}) values for these drugs were determined from either Eadie-Hofstee plots or best one-site computer fits to the data obtained from single neurons under standard test conditions using 10 μM GABA (Table 1). In addition, numerous tests performed on individual neurons with single concentrations of potentiating drugs in their half-maximal range (CDPX, $n = 48$ neurons; FNZM, $n = 21$; clonazepam, $n = 22$; and Ro15-1788, $n = 8$) yielded results entirely consistent with those predicted from the dose-response curves described above.

BDZs enhance the GABA response of CNS neurons by decreasing the EC_{50} for GABA without changing the maximum cellular response to GABA (23). To examine the mechanism by which benzodiazepines modulate ganglionic receptors, multibarrel pipettes were used to generate complete GABA dose-response curves for single neurons in the presence and absence of prior exposure to CDPX. The effect of CDPX was to shift the GABA dose-response curve to the left, i.e., to increase the apparent affinity for GABA without changing the maximum response to GABA (Fig. 2). For five neurons tested in this manner, prior exposure to 100 μM CDPX resulted in a 220 ± 10% decrease in the EC_{50} for the GABA response. A similar 2-fold shift in the GABA dose-response curve was obtained when data from 23 additional neurons tested with one to three concentrations of GABA were used to generate composite curves with and without CDPX (data not shown).

Ro5-4864 (chlorodiazepam) that was initially thought (24) to bind only to nonneuronal BDZ binding sites of the type present in mitochondrial membranes (7, 8). More recently, micromolar concentrations of Ro5-4864 have been shown to inhibit the GABA response of mammalian CNS neurons (25) and may act through the picrotoxin binding site (a site distinct both from the GABA and BDZ binding sites) on the $GABA_A$ receptor (26). On chick ciliary ganglion neurons, as in the mammalian CNS, Ro5-4864 directly inhibits GABA responses (Fig. 3, *open circles*). The results from 11 neurons tested in this manner yielded 23 ± 2% inhibition at 300 nM and 61 ± 4% at 10 μM

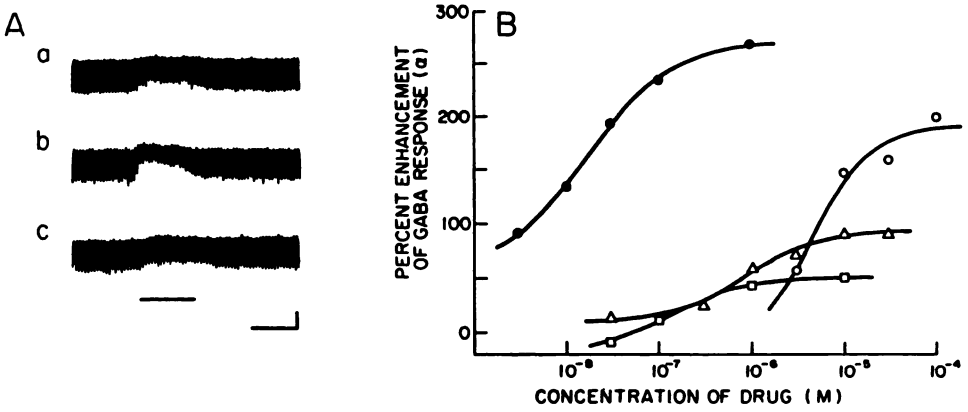

Fig. 1. BDZ agonists and Ro15-1788 enhance the GABA response of chick ciliary ganglion neurons. A, A single ciliary ganglion neuron was tested for responses to 10 μM GABA (*a*) prior to, (b) immediately following, and (c) after recovering from exposure to 100 μM CDPX for 10 sec. In this example, $\alpha = 296$. The record shows membrane potential changes in response to injected constant hyperpolarizing current pulses. The breaks in the record correspond to 45-sec wash periods. Resting potential, −56 mV. Culture age, 5 days. *Calibration bars*, horizontal, 2 sec; vertical, 5 mV. *Isolated horizontal bar*, duration of GABA application. B, Concentration dependence for enhancement of the GABA response by FNZM (●), clonazepam (△), CDPX (○) and Ro15-1788 (□), each tested on individual neurons. The test compound was applied to the neuron for 10–15 sec immediately preceding the challenge with 10 μM GABA. The results are expressed as the per cent enhancement of the basal GABA response (α). The *solid lines* represent the best theoretical one-site fit to the data as determined by nonlinear least squares analysis. EC_{50} estimates of 18 nM, 851 nM, 124 nM, and 4.5 μM and α_{max} values of 272, 95, 51, and 193 were obtained for FNZM, clonazepam, Ro15-1788, and CDPX, respectively, in these examples.

TABLE 1

Efficacies (α_{max}) and potencies (EC_{50}) for enhancement of the GABA response of chick ciliary ganglion neurons by BDZs and an imidazodiazepine

Dose-response curves for each compound were obtained from single neurons and values of α_{max} and EC_{50} were estimated by either nonlinear least squares analysis or Eadie-Hofstee analysis. Values are expressed as the mean ± standard error for n neurons.

Potentiating drug	EC_{50}	α_{max}	n
FNZM	22 ± 5 nM	160 ± 60	3
Ro15-1788	250 ± 70 nM	80 ± 20	4
Clonazepam	1.1 ± 0.3 μM	100 ± 3	3
CDPX	4.6 ± 0.5 μM	140 ± 30	5

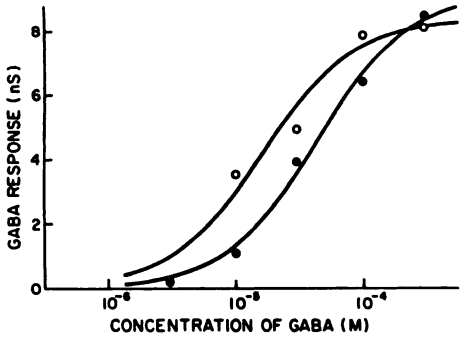

Fig. 2. CDPX shifts the GABA dose-response curve to a higher affinity for GABA. A single neuron was challenged with the indicated concentrations of GABA with (○) and without (●) a 10-sec exposure to 100 μM CDPX immediately before the test, and yielded EC_{50} values of 20 and 44 μM, respectively. Resting potential, −54 mV. Culture age, 5 days.

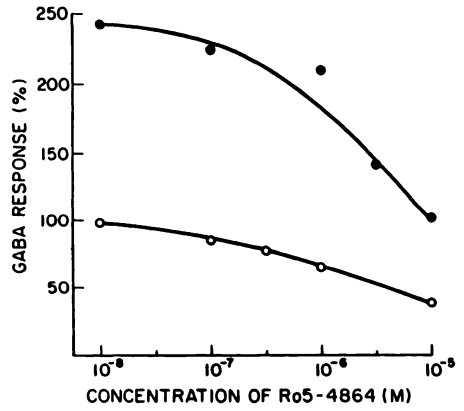

Fig. 3. Ro5-4864 inhibits the GABA response of ciliary ganglion neurons both in the presence and absence of CDPX. GABA responses were determined for single neurons immediately after application of the indicated concentrations of Ro5-4864 either alone (○) or together with 100 μM CDPX (●) for 10–15 sec. Results are expressed as a percentage of the basal GABA response. Resting potentials, −62 and −60 mV, respectively. Culture ages, 6 and 7 days, respectively.

Ro5-4864. Ro5-4864 also reduced the GABA response enhanced by CDPX (e.g., Fig. 3, *closed circles*) by 25 ± 5% at 1 μM (n = 3) and 55 ± 3% at 10 μM (n = 3).

[^{3}H]FNZM binding. [^{3}H]FNZM is a useful ligand for identifying $GABA_A$ receptors in the CNS (27, 28). The finding here that $GABA_A$ receptors on chick ciliary ganglion neurons can be modulated by BDZs in a manner similar to CNS receptors indicates that the ganglionic receptors have BDZ binding sites that may also be identifiable by [^{3}H]FNZM binding. Specific binding of [^{3}H]FNZM was observed with membrane homogenates prepared from embryonic chick ciliary ganglia. The binding was time dependent and reached equilibrium within 20 min at 4° (Fig. 4A). The binding was reversible inasmuch as dilution of the reaction mixture after equilibrium was achieved led to a subsequent time-dependent reduction in the amount of specific binding. The inferred rate of dissociation ($t_{1/2}$, 3–5 min) indicated that no more than 10% of the specific binding was lost during the normal wash procedure for the assay (data not shown). The amount of specific binding was directly proportional to the amount of protein in the assay over the range tested (Fig. 4B). The concentration dependence of [^{3}H]FNZM binding is shown in Fig. 5. Scatchard analysis of three such experiments was consistent with a single class of specific binding sites with a mean apparent K_D of 34 ± 6 nM and a mean B_{max} of 145 ± 26 fmol/mg of protein. The K_D for [^{3}H]FNZM binding is similar to the EC_{50} for FNZM enhancement of receptor activation by GABA (Fig. 1; Table 1), consistent with the BDZ binding site detected here biochemically being the same as the site at which BDZs modulate $GABA_A$ receptor function.

Two additional lines of evidence support the conclusion that the [^{3}H]FNZM binding observed in ciliary ganglion homoge-

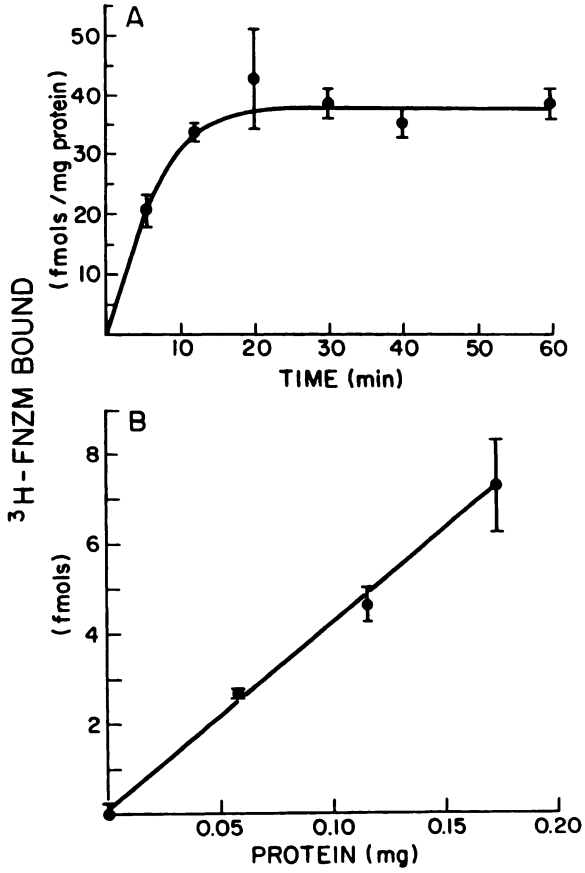

Fig. 4. Dependence of [^{3}H]FNZM binding on time of incubation and amount of membrane homogenate protein. Specific binding of [^{3}H]FNZM in ciliary ganglia membrane homogenates was determined under standard conditions, varying either the time of incubation (A) or the amount of membrane homogenate protein (B) as indicated. The [^{3}H]FNZM concentration was 17.5 and 18.5 nM in A and B, respectively. Total and nonspecific binding in the plateau region in A were 636 ± 16 (10 determinations) and 344 ± 13 (eight determinations) cpm/reaction mixture, respectively. In B the levels were 688 ± 13 (two determinations) and 390 ± 16 (two determinations) cpm/10 ciliary ganglion equivalents, respectively. Similar results were obtained in a second experiment.

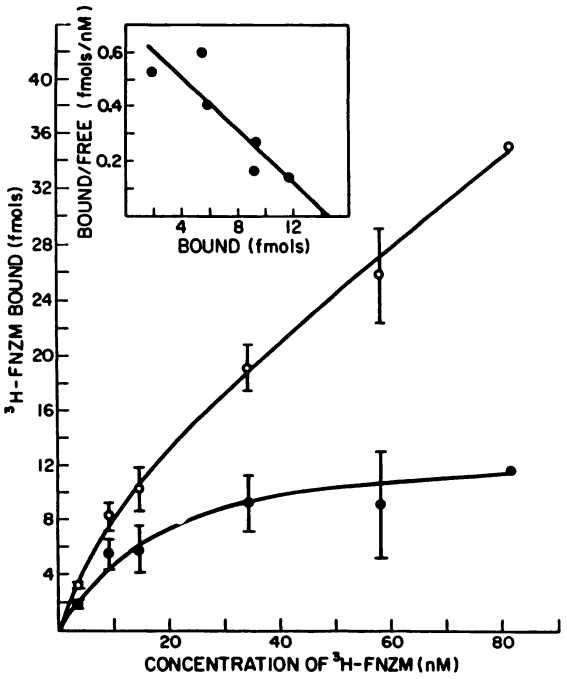

Fig. 5. Concentration dependence of [^{3}H]FNZM binding. Total (○) and specific (●) binding of [^{3}H]FNZM (fmol/reaction mixture) are shown at the indicated concentrations. Scatchard analysis of the data (*inset*) indicated 105 fmol of [^{3}H]FNZM binding sites per mg of protein (0.14 mg of protein/reaction mixture) with an apparent equilibrium dissociation constant (K_D) of 21 nM for this example. The *line* in the *inset* was fit by linear regression, and the resulting binding parameters were used to fit the curve through the data points for specific binding. The specific cpm obtained at the two highest concentrations of [^{3}H]FNZM shown were 674 ± 140 (three determinations). *Points* represent the mean of duplicate determinations except for 83 nM [^{3}H]FNZM, which was a single determination in this experiment.

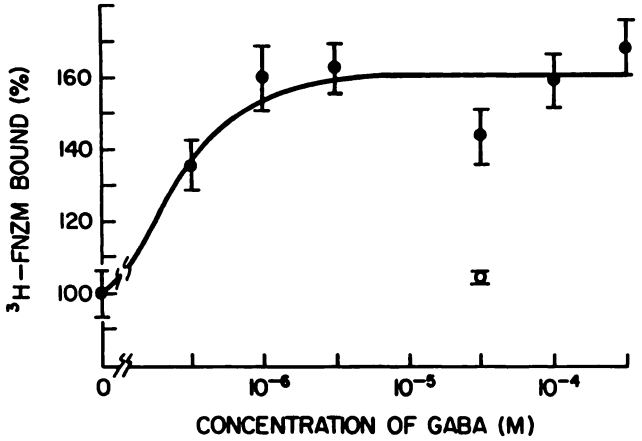

Fig. 6. GABA increases [^{3}H]FNZM binding. Binding reactions were carried out with 6.3 nM [^{3}H]FNZM and the indicated concentrations of GABA in the presence (○) or absence (●) of 100 μM (+)-bicuculline. The results are expressed as a percentage of the [^{3}H]FNZM specific binding obtained in the absence of GABA (19 fmol/mg of protein).

nates is associated with $GABA_A$ receptors. The first applies the finding that GABA enhances the affinity of CNS $GABA_A$ receptors for BDZs (29–32). In ciliary ganglion membrane homogenates, GABA increased specific [^{3}H]FNZM binding (Fig. 6), and at 30 μM yielded a mean increase in [^{3}H]FNZM (6–7 nM) binding of 49 ± 10% in two experiments. Similar results were obtained in three additional experiments in which determinations were made at single GABA concentrations of either 20 μM or 100 μM. The GABA effect was specific inasmuch as bicuculline at 100 μM, which alone had no effect on binding, completely prevented the GABA-mediated enhancement of [^{3}H]FNZM binding (Fig. 6).

The second approach was pharmacological. The ability of compounds to reduce [^{3}H]FNZM binding was compared with their ability to alter $GABA_A$ receptor function. Clonazepam at 0.5 μM inhibited 27 ± 13% (n = 2) of the [^{3}H]FNZM binding obtained at 10^{-8} M, and at 5 μM inhibited 86 ± 3% (n = 3). These values suggest an IC_{50} for clonazepam binding at the [^{3}H]FNZM site (10^{-6} M) that is comparable to the EC_{50} for clonazepam enhancement of the GABA response (Table 1). Ro5-4864 reduced [^{3}H]FNZM binding in a complex manner that did not conform to the expectations of a simple bimolecular reaction (Fig. 7; n_H = 0.36). Nonetheless, Ro5-4864 displayed a similar concentration dependence for inhibition of [^{3}H]FNZM binding and for inhibition of the GABA response obtained both in the presence and absence of CDPX. The findings provide further support for the contention that the [^{3}H]FNZM binding site in membrane homogenates is the site at which BDZs influence GABA activation of the $GABA_A$ receptor on chick ciliary ganglion neurons.

Spinal cord neurons. The results obtained here with chick ciliary ganglia are consistent with the modulation by BDZs of $GABA_A$ receptors present on peripheral neurons and support the use of [^{3}H]FNZM binding to identify the receptors. In most respects the ganglionic receptors appear similar to CNS receptors. In some respects they appear to differ (see Discussion). One potential difference arises from a report that Ro5-4864 has no effect on the GABA response in cultures of chick spinal cord neurons (33), whereas inhibition of the GABA response was observed in the present study with ciliary ganglion neurons. To compare the two cell types directly, spinal cord neurons were grown in culture and tested for Ro5-4864 effects on the

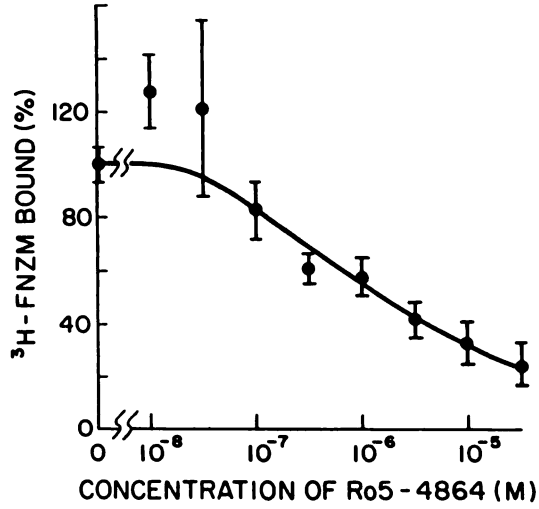

Fig. 7. Ro5-4864 inhibition of [^{3}H]FNZM binding. Membrane homogenates were assayed for [^{3}H]FNZM binding at 18 nM in the presence of the indicated concentrations of Ro5-4864. Results are expressed as a percentage of binding obtained in the absence of Ro5-4864 (29 ± 7 fmol/mg of protein). Each determination represents the mean ± standard error of three separate experiments in duplicate. Binding values in the presence of 10 and 30 nM Ro5-4864 are not significantly different from binding in the absence of Ro5-4864 as determined by the Student's t test applied either to compiled means or to means calculated for individual experiments.

GABA response. The results show that Ro5-4864 inhibits the GABA response of spinal cord neurons in a manner similar to that of the GABA response of ciliary ganglion neurons (Fig. 8). Mean values of 23 ± 5% and 64 ± 3% were obtained for the inhibition achieved by 300 nM and 10 μM Ro5-4864, respectively ($n = 4$).

Discussion

The major findings reported here are that $GABA_A$ receptors on chick ciliary ganglion neurons can be modulated by BDZs and that a radiolabeled BDZ can be used to characterize the receptors in ganglion homogenates. In both respects the ganglionic receptor is similar to the $GABA_A$ receptor mediating inhibitory synaptic transmission in the vertebrate CNS.

The effect of BDZs on GABA responses of ciliary ganglion neurons can be accounted for by a shift of the GABA dose-response curve to lower concentrations, as is true for BDZ modulation of $GABA_A$ receptors in the CNS (23). CDPX, FNZM, clonazepam, and Ro15-1788 all increased submaximal GABA responses of the neurons in a reversible dose-dependent manner. No increase in the maximal GABA response was observed in the presence of BDZs. It might be argued that BDZs, nonetheless, increase the maximal response to GABA and that the increase is obscured by an increased rate of GABA receptor desensitization brought on by BDZs (34). This explanation is unlikely because little difference is observed in the rates of desensitization with and without BDZs at high GABA concentrations in the present studies (data not shown).

Several lines of evidence support the conclusion that the [^{3}H]FNZM binding sites detected in ganglion membrane homogenates represent $GABA_A$ receptors. Scatchard analysis of the binding is consistent with the presence of a single population of high affinity sites with a K_D of 34 nM, which is in good agreement with the EC_{50} of 22 nM for FNZM-mediated enhancement of the GABA response on ciliary ganglion neurons. GABA (≥1 μM) increases [^{3}H]FNZM binding to a similar maximal extent in chick ciliary ganglion (50–60%) and CNS (20–80%) membrane homogenates (20, 35, 36). Clonazepam and Ro5-4864 each reduce [^{3}H]FNZM binding with the same concentration dependence that they exhibit in modulating the function of $GABA_A$ receptors. Mammalian nonneuronal BDZ binding sites of the type thought to be associated with mitochondrial membranes (7, 8), and not with the $GABA_A$ receptor, have a K_D for [^{3}H]FNZM 10-fold higher than does the CNS $GABA_A$ receptor and a low affinity for clonazepam (37), as described here for the ganglionic $GABA_A$ receptor. The mammalian nonneuronal sites, however, have an affinity for Ro5-4864 that is approximately 100-fold higher than that of ganglionic $GABA_A$ receptors. If ciliary ganglion homogenates contain nonneuronal BDZ binding sites of the mammalian type, they are either low in number or low in affinity and went undetected in the present studies. The Scatchard analysis of [^{3}H]FNZM binding could not exclude the presence of a small second population of binding sites.

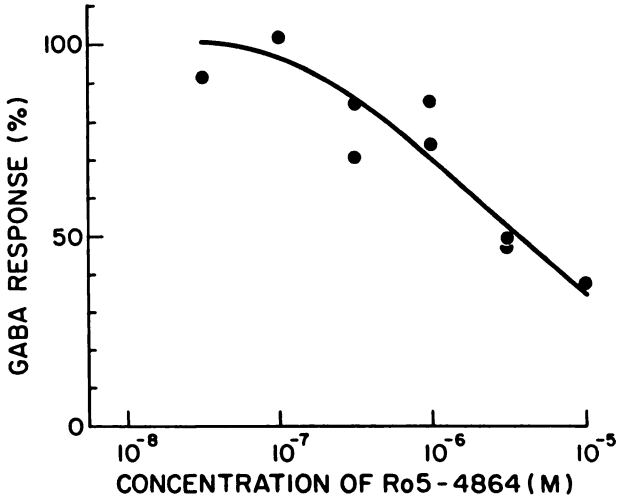

Fig. 8. Ro5-4864 inhibits the GABA response of chick spinal cord neurons. GABA responses were determined from a single neuron after 10–15 sec exposure to the indicated concentrations of Ro5-4864. Results are single determinations expressed as a percentage of the basal GABA response. Resting potential, −50 mV. Culture age, 8 days.

The avian ganglionic $GABA_A$ receptor described here differs in several respects from previously characterized avian CNS receptors. $GABA_A$ receptors from the avian CNS bind [^{3}H]FNZM with a K_D of 2–6 nM (20, 27, 36, 38), values at least 5-fold lower than the K_D of 34 nM found here for ganglionic receptors. The IC_{50} value for clonazepam is at least 100-fold higher for ganglionic receptors (1 μM) than for central receptors (approximately 10 nM; Ref. 33). The EC_{50} values for the effects of FNZM, clonazepam, and CDPX determined here for ganglionic receptors differ by 3–8-fold from those reported for chick spinal cord receptors (19, 23). CNS receptors are thought to undergo a steep temperature dependence in BDZ binding, which could account in part for the difference in BDZ affinities inferred from binding studies at 4° and functional studies at 37° (19, 20, 27). No large temperature dependence of BDZ binding was apparent in the present studies on ganglionic $GABA_A$ receptors comparing binding at 4° with modulation at 37°. Lastly, Ro5-4864 at concentrations that we find decrease [^{3}H]FNZM binding to ganglionic receptors has been reported to have no effect on binding to mammalian and avian CNS receptors (27, 28) and bovine adrenal chromaffin cells (10).

Some of these differences may arise from difficulties inherent in the techniques; at least 2-fold variation is encountered among experiments in determining K_D values for [^{3}H]FNZM binding in ganglionic membrane homogenates. Similar variability has been reported for BDZ binding to locust supraoesophageal ganglion membranes (39) and may be due to problems in working with small amounts of tissue. In addition, EC_{50} values may be influenced by shifts in the dose-response curves produced by leak artifacts inherent in a multibarrel puffer technique. A previous report that Ro5-4864 fails to block GABA responses of chick CNS neurons in culture (33) posed a potential conflict with the present findings because the compound does inhibit ganglionic $GABA_A$ receptor function. Testing the compound on both types of neurons in the experimental paradigm used here, however, revealed identical results, i.e., a partial blockade of the GABA response by Ro5-4864, supporting the view that CNS and ganglionic receptors are similar in this respect. Nonetheless, differences may exist between $GABA_A$ receptors on ganglionic and CNS neurons. It is unlikely that the large differences between the EC_{50} values for FNZM and clonazepam on ganglionic and CNS receptors or the difference in Ro5-4864 inhibition of [^{3}H]FNZM binding can be accounted for by the kinds of considerations described above. In some of these respects the ganglionic BDZ receptors resemble certain

insect BDZ binding sites thought to be associated with GABA receptors (39, 40).

The basic features of $GABA_A$ receptors in the chick ciliary ganglion are similar to those of $GABA_A$ receptors in the CNS. The receptors may carry out similar functions, although a source of agonist for the ganglionic receptors has yet to be identified. The pharmacological differences reported here between ganglionic and CNS $GABA_A$ receptors suggest that either the receptors are encoded by distinct, although similar, genes or that some aspects of receptor function are determined by posttranscriptional events that differ among cell types.

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