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Effect of Benzodiazepines on Cyclic GMP Formation in Rat Cerebellar Slices

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SMITH, C. C., M. E. LEWIS AND J. F. TALLMAN. *Effect of benzodiazepines on cyclic GMP formation in rat cerebellar slices*. PHARMAC. BIOCHEM. BEHAV. **16**(1) 29–33, 1982.—Cyclic 3',5'guanosine monophosphate (cGMP) levels, stimulated in brain slices by ouabain, potassium, and sodium azide depolarization, have been found to be inhibited in a dose-dependent fashion by both behaviorally active and inactive benzodiazepines. ED_{50}'s for these benzodiazepines bear no relation to potencies established from receptor membrane binding studies; however, these values do correspond to ED_{50}'s calculated for several known membrane stabilizing agents tested in this system. The concentration of [^{3}H]flunitrazepam necessary to saturate the benzodiazepine receptor in identical slice preparations is considerably lower than that needed to initiate inhibition of depolarization-induced cGMP stimulation. Some of the reported *in vivo* actions of benzodiazepines on cGMP formation may be the result of membrane stabilizing actions of the benzodiazepines or mediated indirectly.

Cyclic GMP Benzodiazepines GABA Benzodiazepine receptors Brain slices Diazepam Ouabain

RECENT investigations have implicated GABA-ergic mechanisms in the actions of the benzodiazepines [8, 16, 18]. In particular, the ability of GABA and its analogues to modulate the affinity of the binding site for benzodiazepines [6] points out an intimate relationship, probably allosteric, between the benzodiazepine receptor and GABA receptor. Electrophysiological [8,17] and behavioral [14] data support the close association of these receptors, and intracellular recording data indicate that the action of GABA and benzodiazepines is related to the activity of a chloride ionophore [2,19]. However, little information exists about events subsequent to the opening of the anion channel. In the past, the inhibition of cyclic GMP formation by benzodiazepines has been suggested as a possible mediator of their actions [3]; the design of these experiments was indirect and a series of benzodiazepines was not examined.

In this paper, we describe experiments using brain slices which attempt directly to correlate the occupancy of the benzodiazepine binding sites on brain slices with the ability of these drugs to inhibit cyclic GMP formation stimulated by a number of depolarizing agents. No correlation could be demonstrated between receptor occupancy and the ability to inhibit cyclic GMP formation.

METHOD

In these experiments, male Sprague-Dawley rats (200–250 g) were used. The animals were killed by decapitation, and cerebella removed and placed in ice-cold Krebs-Ringer buffer, pH 7.4, containing 124 mM sodium chloride, 5 mM potassium chloride, 0.8 mM calcium chloride, 1.3 mM magnesium chloride, 10 mM dextrose, 1.4 mM potassium phosphate, 26 mM sodium carbonate, 5 mM isobutylmethylxanthine, and gassed with 95% oxygen and 5% carbon dioxide.

Tissue samples were then cut into 0.25 mm-thick slices using a Brinkmann-McIlwain tissue chopper, rotated 45°, cut again, suspended in 50 volumes of fresh Krebs-Ringer buffer, and placed in a shaker bath at 37°C (70 oscillations per min) for 75 min. Buffer was changed every 25 min (with freshly gassed buffer); the final resuspension was to 10 volumes.

Cyclic GMP Stimulation

A typical incubation was carried out for 30 min in a 250 μl total volume of Krebs-Ringer buffer at 37°C, containing 0.2–0.5 mg protein per assay. The reaction was terminated by placing the tubes in boiling water. Tubes were centrifuged, and the supernatant was withdrawn and assayed for cyclic GMP content using a radioimmunoassay method [15]. The recovery of cyclic GMP is greater than 90% for this method. The brain pellets were suspended in sodium hydroxide, sonicated, and reserved for protein determination [10]. All incubations were done in triplicate.

Benzodiazepine Binding

Aliquots of cerebellar slices, prepared as above, were added to incubations of Krebs-Ringer buffer (0.4–0.75 mg

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protein per assay) to a total incubation volume of 250 μl. [^{3}H]Flunitrazepam (84.8 Ci/mmol, New England Nuclear) was used as the radioactive ligand. Incubations were run for 30 min at 0–4°C or at 37°C and were stopped by the addition of 1 ml ice-cold buffer and immediate centrifugation (Beckman Microfuge). The supernatant was removed and the slices resuspended in 1 N sodium hydroxide and sonicated. Aliquots were taken to determine binding to the pellet by liquid scintillation counting and the protein content was determined [10]. All assays were done in triplicate; binding in the presence of 1×10^{-5} M clonazepam is defined as nonspecific binding. Binding of [^{3}H]flunitrazepam to membranes was determined as previously described [6].

Ouabain Binding

Cerebellar slices, prepared as before, were suspended in 50 volumes of buffer containing 50 mM Tris (pH 7.7), 5 mM magnesium chloride and 125 mM sodium chloride. The slices were permitted to settle: the supernatant was removed and the slices resuspended to 10 volumes (w/v). Assays were done in triplicate in a 200 μl total incubation volume, which contained [^{3}H]ouabain and 1.25 mM adenosine triphosphate (ATP). The assay was carried out at 37°C for 30 min, and was terminated by centrifugation in a microfuge, aspirating the supernatant and washing the pellet once superficially with ice-cold Tris buffer which contained 15 mM KCl and 5 mM $MgCl_2$. The pellet was suspended in sodium hydroxide and sonicated. Aliquots were taken to determine binding and protein [10]. Ouabain binding is ATP dependent [1]; nonspecific binding was determined by deletion of ATP from the assay.

All benzodiazepines utilized in these studies were supplied by Hoffmann-La Roche (Nutley, NJ). Other chemicals used include adenosine 5′-triphosphate (sodium salt), propranolol hydrochloride, tetracaine hydrochloride, hexobarbital, mepacrine and chloroquine diphosphate salt (Sigma Chemical); ouabain (California Corporation for Biochemical Research); 7-chloro-4-hydrazinoquinoline and 3-isobutyl-l-methylxanthine (IBMX) (Aldrich); [^{3}H]flunitrazepam (New England Nuclear); [^{3}H]ouabain and cyclic GMP radioimmune assay kit (Amersham).

RESULTS

Data obtained from the addition of increasing concentrations of ouabain, potassium, and sodium azide to incubations of rat cerebellar brain slices confirm earlier reports which indicate that depolarization-induced stimulation of cyclic GMP responds in a dose-dependent manner (Fig. 1). Similar numerical increases in cyclic GMP occurred for each of these agents; the basal level of cyclic GMP ranged in the experiments reported here from 11.0 to 18.0 pmol/mg protein.

The addition of benzodiazepines to brain slice incubations containing either ouabain, potassium, or sodium azide used at their ED_{50}'s was found to inhibit the observed increase in cyclic GMP levels. This response was dose dependent; however, it bore no correlation to the established potencies of the benzodiazepines in displacing binding from brain receptors. RO5-4864, demonstrated to be a relatively inactive compound based upon its inability to displace known active analogs in receptor binding studies [18], was as equally effective in inhibiting cyclic GMP stimulation as other benzodiazepines. Independent of the source of depolarization (ouabain, potassium, or sodium azide), all benzodiazepines exhibited ED_{50}'s in the range of 7.5×10^{-6} to 3.8×10^{-5} M (Table 1).

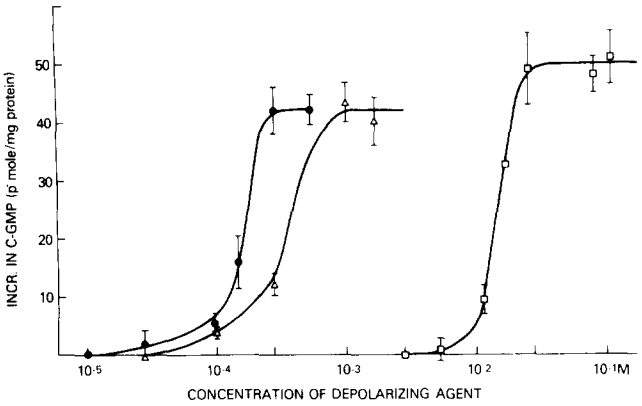

FIG. 1. Dose response curves showing increases in cyclic GMP levels over baseline concentrations in response to sodium azide (●), ouabain (△), and potassium (□) stimulation. Results are the mean of three separate determinations; the vertical bars indicate the standard error of the mean. Assays were carried out as described in Method.

TABLE 1

DOSE REQUIRED FOR 50% INHIBITION OF DEPOLARIZATION-INDUCED STIMULATION OF CYCLIC GMP FORMATION

Addition	Ouabain (μM)	Potassium (μM)	Sodium Azide (μM)
Flunitrazepam	32	7.5	8.9
Flurazepam	37	52	10.5
Diazepam	38	75	10.5
Clonazepam	25	29	30
RO5-4864	20	8.5	9.5

The concentrations of ouabain, potassium and sodium azide utilized were those determined to effect a 50% stimulation of cGMP production, as shown in Fig. 1. The concentrations of benzodiazepines used in determining 50% inhibition of that cGMP stimulation ranged from 10^{-6} M to 10^{-4} M.

To ascertain whether this effect was specific to the benzodiazepines or attributable to a membrane-stabilizing effect, hexobarbital, tetracaine, propranolol, mepacrine, chloroquine, and 7-chloro-4-hydrazinoquinoline were tested against all three depolarizing agents; all were found to be active at 1×10^{-4} M. Dose-response curves using mepacrine, chloroquine, and 7-chloro-4-hydrazinoquinoline revealed that the three compounds had ED_{50}'s in the range of 7.8×10^{-6} to 3.5×10^{-5} M, which were independent of the depolarizing agent (Table 2). The ED_{50}'s of these compounds compare very closely to those determined for the benzodiazepines.

Utilizing cerebellar slices prepared in the same manner, binding studies were carried out with [^{3}H]flunitrazepam and [^{3}H]ouabain to determine if an active site of cyclic GMP modulation might be on or regulated by one of these recep-

TABLE 2

DOSE REQUIRED FOR 50% INHIBITION OF DEPOLARIZATION-INDUCED STIMULATION OF CYCLIC GMP FORMATION USING MEMBRANE-STABLIZING AGENTS

Addition	Ouabain (μM)	Potassium (μM)	Sodium Azide (μM)
Mepacrine	26	16	20
Chloroquine	26	7.8	31
7-Chloro-4-hydrazinoquinoline	35	20	35

Ouabain, potassium, and sodium azide were used at concentrations which induced a 50% stimulation of cGMP production, as shown in Fig. 1. The concentrations of membrane stabilizing agents utilized ranged from 10^{-6} M to 10^{-4} M.

tors. Specific binding of [^{3}H]flunitrazepam was obtained and, as determined by Scatchard analysis, the maximum binding of flunitrazepam was 0.54 pmol/mg protein. The affinity of these sites was 2.5 nM. In determining the reliability of this methodology as an indicator of specific binding, a side-by-side comparison was done using prepared membranes. Results indicate a greater apparent number of [^{3}H] flunitrazepam binding sites in brain slice than in homogenates; the affinity of these sites for the ligand was slightly higher in slices than in membranes. The same slice study carried out at 37° resulted in an affinity of 5.9 nM, approximately half of that at 0–4°; the number of sites was unchanged. The binding of ouabain was 1.85 pmol/mg protein, and the affinity of these sites was 43.5 nM (Figs. 2 and 3).

Ouabain, found active as a stimulator of cyclic GMP production at a concentration of 1×10^{-4} M, reached a maximum level of stimulation at 1×10^{-3} M. Flunitrazepam was effective in inhibiting this response in concentrations from 1×10^{-5} to 1×10^{-4} M (Fig. 4). It is apparent from these data that ouabain binding sites associated with the sodium-potassium ATP-ase molecule should be saturated prior to ouabain's effect upon cyclic GMP stimulation and that the benzodiazepine binding sites should also be saturated prior to the initiation of the inhibitory response to benzodiazepines.

Competitive binding studies in which clonazepam, flurazepam, flunitrazepam, diazepam, and RO5-4864 were added in doses from 1×10^{-9} to 1×10^{-5} M confirmed a dose-dependent ability to displace the radioactive ligand. RO5-4864 was inactive in displacing [^{3}H]flunitrazepam binding. Ouabain, added in concentrations from 1×10^{-9} to 1×10^{-4} also failed to elicit an effect upon flunitrazepam binding (Fig. 4). Since GABA has been shown to modulate the affinity of the binding site for the benzodiazepines in well washed membranes, we examined the effects of GABA and muscimol on binding to slices. We were unable to demonstrate any clear alteration in binding of [^{3}H]flunitrazepam, probably due to the large amount of GABA present in the slices; similarily in doses up to 10^{-5} M, bicuculline did not reverse the effect of endogenous GABA on binding. There were no effects of GABA-ergic agonists or antagonists on cyclic GMP formation.

The effect of benzodiazepines upon ouabain binding was assessed utilizing flunitrazepam over the range of 1×10^{-9} to 1×10^{-4} M against [^{3}H]ouabain at a concentration of 25 nM.

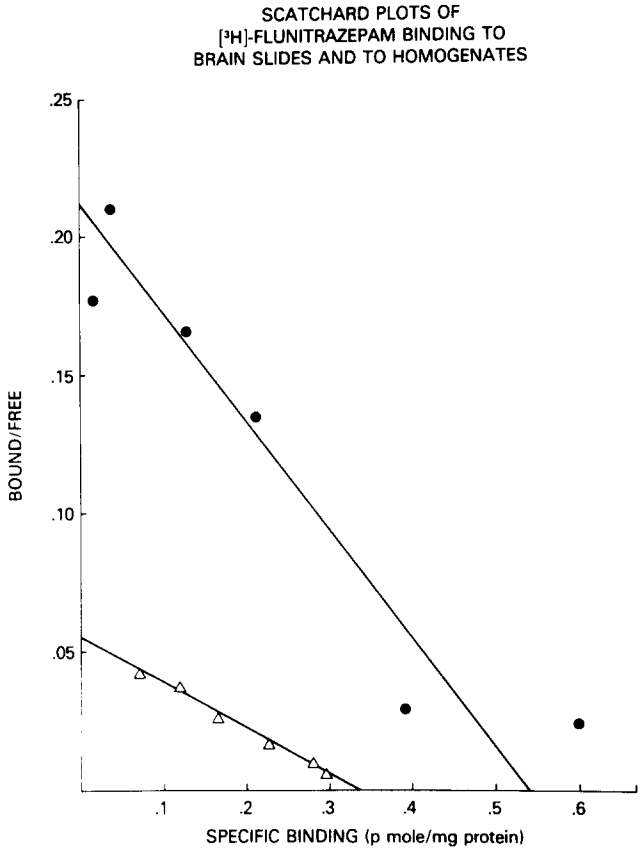

FIG. 2. Scatchard analysis of [^{3}H]flunitrazepam binding to cerebellar membranes (△) and to cerebellar brain slices (●), prepared as described in the text. The concentration of [^{3}H]flunitrazepam was varied from 0.78 nM to 200 nM. Data in each case represents the average of three determinations.

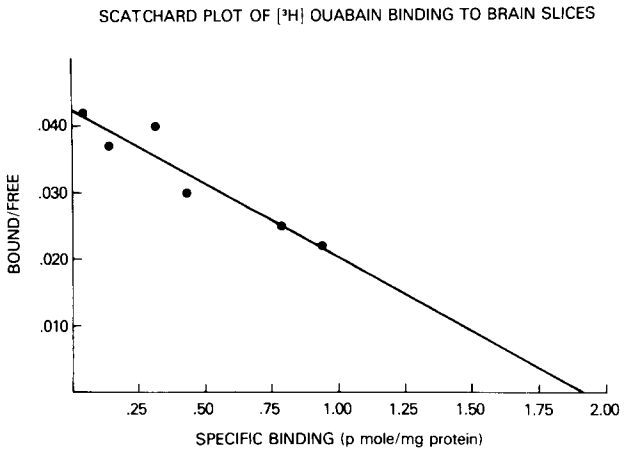

FIG. 3. Scatchard analysis of [^{3}H]ouabain binding to cerebellar brain slices, prepared as described in Method. Concentrations of [^{3}H]ouabain utilized varied from 4.8 nM to 210 nM. Each point represents the mean of three determinations.

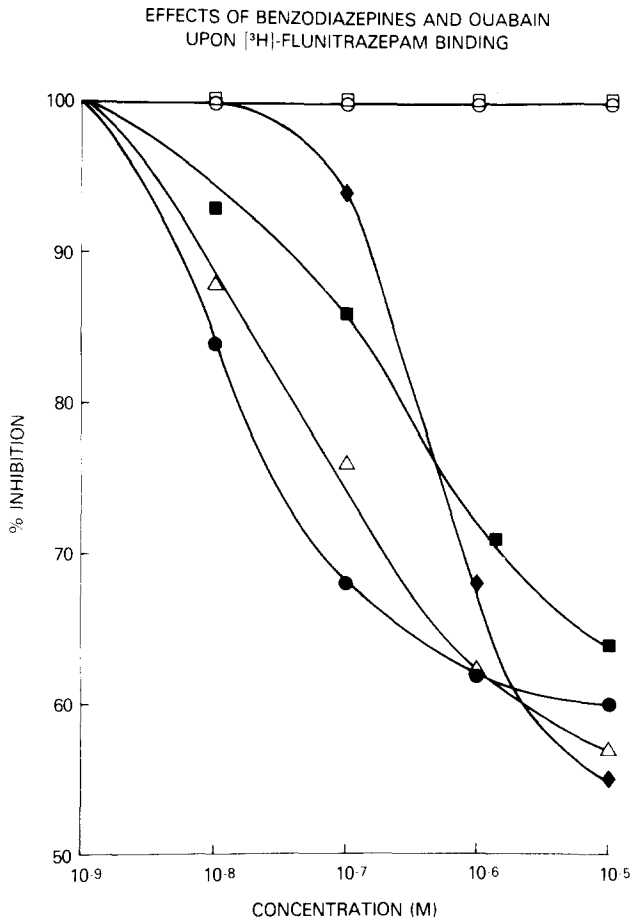

FIG. 4. Competitive inhibition by clonazepam (●), flunitrazepam (△), diazepam (◆), flurazepam (■), RO5-4864 (○), and ouabain (□) of [^{3}H]flunitrazepam in cerebellar brain slices. Inhibition values are expressed as the percent inhibition, and represent the mean of triplicate determinations. The concentration of [^{3}H]flunitrazepam used in these determinations was 25 nM.

No effects of benzodiazepines were observed. Mepacrine, also observed to decrease cyclic GMP stimulation, similarily had no effect upon ouabain binding.

DISCUSSION

The depolarization-induced stimulation of cyclic GMP by ouabain, in inhibiting ($Na^+ + K^+$)Atp'ase, and by potassium support the hypotheses [4] that cyclic GMP stimulation is, in this instance, a function of a general membrane depolarization. The stimulation of cyclic GMP levels by sodium azide has been attributed to the direct stimulation of particulate guanylate cyclase; however, general toxic effects of this chemical cannot be ruled out as a possible cause [9]. All three agents responded in a dose-dependent manner, and it was apparent that the absolute net increase of cyclic GMP levels was about the same for each of these agents.

Results of recent investigations indicate a close correlation between the GABAergic purkinje network of the cerebellum and changes in levels of cyclic GMP [4, 11–13]. Biochemical and electrophysiological studies have clearly demonstrated an allosteric relationship between the benzodiazepine receptor and GABA receptor [6,14]. It was not, therefore, altogether surprising to find that the addition of benzodiazepines would result in decreases in cyclic GMP levels. However, upon examination of the ED_{50}'s of the benzodiazepines tested against ouabain, sodium azide, and potassium stimulation, it was apparent that all values were within the same range (7.5×10^{-6} to 5.2×10^{-5} M). It was clear in this experiment also, that the activity of the relatively inactive RO5-4864 (as determined in receptor binding studies) was similar to known, centrally active benzodiazepines. If the increase in cyclic GMP levels was directly mediated through the benzodiazepine-GABA receptor system, RO5-4864 should have been inactive.

Mepacrine, chloroquine, 7-choloro-4-hydrazinoquinoline, hexobarbital, tetracaine, and propranolol, which all possess membrane stabilizing activity, were also tested. Results indicate that all compounds were active in reducing cyclic GMP stimulation. Of those tested over the same concentration range as the benzodiazepines (Table 2), all had ED_{50}'s in the same range, and all values appear to be independent of the source of depolarization.

For further comparison, binding studies utilizing [^{3}H]flunitrazepam were done on cerebellar slices prepared in the same manner and with the same buffer. A comparison of the Scatchard plots obtained by this method and by traditional membrane-binding methodologies (Fig. 2) indicated an increase in both affinity and receptor number when using slices. [^{3}H]Ouabain binding was done with brain slices prepared in the same manner; however, a different buffer system was used (same in both homogenate and slice techniques). The results were comparable to those done with [^{3}H]flunitrazepam (Fig. 3).

The concentration of ouabain necessary to fully saturate all sites on the ($Na^+ + K^+$)ATP'ase molecule, as determined from Scatchard plot analysis, is considerably less than that necessary to initiate depolarization. Similarly, it was determined that the concentration of [^{3}H]flunitrazepam necessary to fully saturate all benzodiazepine binding sites was considerably less than the concentration necessary to initiate the inhibition of cyclic GMP stimulation.

The effectiveness of an inactive benzodiazepine (RO5-4864) in inhibiting the stimulation of cyclic GMP, the similarities of the ED_{50}'s of the benzodiazepines and the membrane stabilizing agents, the failure of the benzodiazepines to exhibit potencies corresponding to their established affinities (based upon membrane binding assays), the differences in the concentration ranges between receptor site saturation and the initiation of the inhibition of cyclic GMP stimulation (flunitrazepam), support the premise of benzodiazepines acting as membrane-stabilizing agents relative to the effect of benzodiazepines on cyclic GMP formation in slices.

Competitive binding studies further support this conclusion. None of the benzodiazepines tested were able to inhibit the binding of [^{3}H]ouabain to its receptor; neither was ouabain able to inhibit the binding of [^{3}H]flunitrazepam to its receptor. It was observed, as a check of our methodology, that RO5-4864 was inactive in attempting to displace [^{3}H]flunitrazepam.

Thus, some of the *in vivo* actions of benzodiazepines on cyclic GMP formation [3] may be a result of nonspecific actions of the benzodiazepines at the membrane level in high doses or mediated quite indirectly. The data presented here cannot definitively answer this question as the experiments are not exactly analogous to the *in vivo* paradigm and may

proceed by a different mechanism, eg. membrane stabilization. However, the same type of membrane stabilization obtains *in vivo* and may be responsible for some of the biological effects of the benzodiazepines. In this regard, agents which cause convulsions may raise cyclic GMP levels [4]; the benzodiazepines may block these increases not by blocking cyclic GMP formation directly but rather by blocking the seizure. Further investigations are required to demonstrate *in vitro* systems for investigation of benzodiazepine receptor mediated events in brain.

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