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Regional Cyclic Nucleotide Phosphodiesterase Activity in Cat Central Nervous System: Effects of Benzodiazepines (37820)

COLIN DALTON, HERMAN J. CROWLEY, HERBERT SHEPPARD, AND WILLIAM SCHALLEK

Departments of Cell Biology and Pharmacology, Research Division, Hoffmann-La Roche, Inc., Nutley, New Jersey 07110

It has been suggested that the antianxiety activity of benzodiazepines may be mediated by cAMP (1). Since this nucleotide is metabolized by cAMP-PD, a drug which inhibits the enzyme would increase the concentration of the nucleotide in the brain. Diazepam has been reported to be an inhibitor of cAMP-PD in rat brain and cat heart (2). Other benzodiazepines are potent inhibitors of this enzyme in rat erythrocyte and dog cerebral cortex (unpublished data, Sheppard and Wiggan). It is therefore of interest to explore the structural requirements in the benzodiazepine series for PD inhibition and to compare the potencies of various benzodiazepines with those of known PD inhibitors.

Evidence has been reported for a selective action of chlordiazepoxide hydrochloride on the hippocampus of the immobilized cat (3). For this reason, we explored the possibility of a correlation between PD inhibition and regional pharmacological activity in the CNS of the cat.

It will be shown that the benzodiazepines diazepam and medazepam hydrochloride are potent inhibitors of both cAMP and cGMP phosphodiesterase. Even though PD activity was found throughout the CNS of the cat, no regional specificity with regard to enzyme activity or drug inhibition could be demonstrated.

Methods. Preparation of tissues. Freshly dissected samples were obtained from different regions of the cat brain. The cats were anesthetized with pentobarbital. The brain was perfused with ice-cold saline via the aorta in order to remove blood in the *in vitro* assay; this step was omitted in the *in vivo* test. Rat brains were obtained from animals killed by cervical dislocation. Neuroblastoma cells (NS-20) originated in mice. Tissues were homogenized in water or 10 m*M* Tris buffer (1:10 w/v), pH 7.4, and centrifuged at 105,000*g*. The supernatant was diluted either 1:10 or 1:100 and 10-μl aliquots were used in the enzyme assay for cAMP-PD (4) and the radioassay for cGMP-PD.

Assay for cAMP phosphodiesterase activity. This assay involves making phosphodiesterase the rate-limiting step in a sequence of reactions starting with the conversion of cAMP to 5′-AMP and ending with the formation of ATP. ATP is then quantitated by the light generated in the firefly luciferin–luciferase reaction using a Luminescence Biometer.

The substrate concentration was 5 μM; aqueous solutions of the drugs were added directly to the reaction cuvets. The final reaction volume was 150 μl. Drugs were also added to the reaction mixture minus cAMP in order to study blank activity.

Data obtained is expressed as picomoles of cAMP hydrolyzed per milligram of tissue per minute; drug effect is calculated as percent inhibition. Each value represents the mean of four determinations ± the standard error of the mean (SEM). In order to obtain accurate comparisons of potency, some

[1] The following abbreviations are used in this paper: cAMP, cyclic-adenosine 3′,5′-monophosphate; cGMP, cyclic-guanosine 3′,5′-monophosphate; CNS, central nervous system; PD, phosphodiesterase.

Cyclic 3′,5′-AMP	Phosphodiesterase →	5′-AMP
5′-AMP + ATP	Myokinase →	2 ADP
ADP + Phosphoenol pyruvate	Pyruvate kinase →	ATP + Pyruvate
ATP–luciferin	Luciferase →	Adenyloxy–luciferin + light

compounds were tested at many different concentrations; the data obtained were plotted as log function of concentration from which an ID_{50} was obtained by inspection.

Assay for cGMP phosphodiesterase activity. Samples to be assayed for cGMP-PD activity were incubated at 37° with 40 m*M* Tris buffer, pH 7.4, 2 m*M* $MgCl_2$, and 1 μCi [8-^{3}H]cGMP ammonium salt. Substrate concentration was 5 μM; aqueous solutions of the drugs were added directly to the reaction tubes to a total volume of 250 μl. The reactions, which were run in triplicate, were terminated after 15 min by placing the tubes in boiling water for 3 min.

Aliquots (10 μl) of each reaction product were cochromatographed with a carrier mixture of cGMP, GMP, guanosine, and guanine (20 nmoles of each) on Whatmann No. 1 filter paper in an ascending fashion for 16 hr. The solvent system was ethanol–NH_4Ac (1 *M*)–H_2O (5:1:1). The ultraviolet-absorbing areas were cut out and placed in 15 ml toluene, 0.4% butyl PBD, for radioactivity determination in a Nuclear-Chicago Mark II liquid scintillation spectrometer. The amount of radioactivity in the cGMP, GMP, and guanine/guanosine areas was expressed as percent of the total radioactivity of each sample. Enzyme activity, which was calculated on the basis of the disappearance of the labeled cGMP, was expressed as picomoles of cGMP hydrolyzed per milligram of tissue per minute; the drug effects were calculated as percent inhibition.

TABLE I. Activity of Standard cAMP Phosphodiesterase Inhibitors in Various Tissues.

Drug	I_{50} (μM) Cat cortex	Rat brain	Neuroblastoma cell
Papaverine	12	3.5	5.5
Dipyridamole	15	12	1.5
Ro 20–1724	15	28	2.7
Medazepam HCl	18	25	15.0
SQ 20006	100	53	30
Theophylline	200	800	>1000

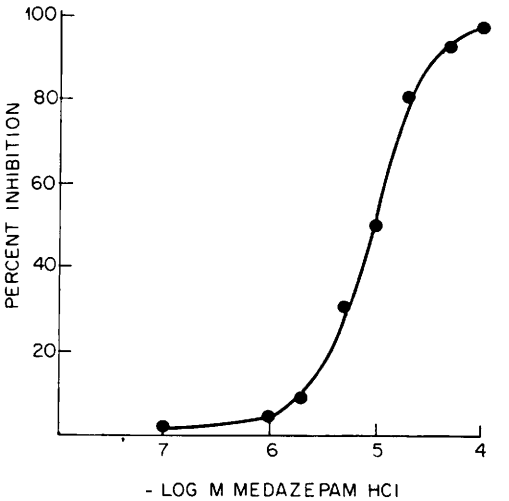

FIG. 1. Effect of medazepam HCl on rat brain cyclic AMP-phosphodiesterase activity. Aliquots of a 105,000*g* supernatant were incubated with 5 μM cAMP and the reaction product detected by the enzymatic method of Weiss *et al.* (4). Data obtained are expressed as picomoles of cAMP hydrolyzed per milligram of tissue per minute. tion and is plotted as a log function of drug The drug effect is calculated as percent inhibiconcentration.

Results. The activity of some known PD inhibitors was tested in supernatants prepared from cat cerebral cortex, rat brain, and mouse neuroblastoma (Table I). The compounds included papaverine (5), dipyridamole (5), Ro 205-1724, 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (6), and SQ 20006, 1-methyl-4-hydrazino-(3,4,b)-pyridine-5-carboxylic acid ethyl ester HCl (7). Also included was medazepam HCl, 7-chloro-2,3-dihydro-1-methyl-5-phenyl-1H-1,4-benzodiazepine HCl. The pharmacology of this compound has been described (8), but it has not previously been tested for PD inhibition. The action of this drug on PD from rat cortex is shown in Fig. 1.

TABLE II. Regional Phosphodiesterase Activity and the Effect of Medazepam HCl in the Cat Brain.[a]

	pmoles/mg tissue/min		% Inhibition with medazepam HCl	
	cAMP	cGMP	cAMP	cGMP
Temporal cortex	171.5 ± 16.0	202	56.2*	41.6
Occipital cortex	164.5 ± 4.3	162	54.2**	36.9
Frontal cortex	104.7 ± 1.9	147	53.1**	31.1
Parietal cortex	100.1 ± 2.4	162	42.8**	21.3
Caudate nucleus	98.8 ± 3.1	63	47.4**	23.0
Cerebellum	96.9 ± 4.1	87	37.8**	27.6
Hippocampus	78.3 ± 2.6	97	46.8**	20.8
Hypothalamus	77.7 ± 3.4	80	55.3**	63.9
Thalamus	67.3 ± 1.9	78	57.4**	50.9
Mesencephalon	54.2 ± 2.2	39	63.6**	50.1
Pituitary	53.2 ± 1.8	23	54.3**	68.5
Olfactory bulb	42.9 ± 2.1	46	58.0**	65.9
Medulla	38.3 ± 1.6	29	58.2**	42.3
Pons	32.8 ± 3.7	33	61.0*	38.8
Spinal cord	26.8 ± 0.7	21	72.0**	56.1

[a] cAMP, 5 μM; Medazepam HCl, 50 μM.
* $P < .01$.
** $P < .001$.

Papaverine, dipyridamole, Ro 20-1724, and medazepam HCl showed approximately the same potency in cat cortex; SQ 20006 and theophylline were less potent. The order of potency of these compounds in rat brain was similar to that in cat cortex. The neuroblastoma cell enzyme appeared to be particularly sensitive to inhibition by all the agents studied except theophylline. Dipyridamole, the most active compound, had an I_{50} of 1.5 μM. Theophylline, which is widely used as an inhibitor of PD, was not very active against any of the enzymes studied.

cAMP-PD and cGMP-PD activity was found throughout the CNS of the cat (Table II). High activity was found in the cortex; lesser activity was found in other regions of the brain, in the spinal cord, and in the pituitary gland. The inhibitory effect of medazepam HCl, which was tested against enzyme prepared from different regions of the CNS, was evident in all tissues.

Other benzodiazepines were studied in the cat cortex, hippocampus, and cerebellum (Table III). All the compounds showed activity in each region. Chlordiazepoxide HCl was weaker than diazepam. The diazepam metabolites *N*-demethyldiazepam, *N*-methyloxazepam, and oxazepam had less activity than the parent compound.

An attempt was made to demonstrate *in vivo* inhibition of cAMP-PD in the cat brain. Diazepam (5 mg/kg) was administered orally to two cats; the brain was dissected after 60 min. No difference in the specific activity of the PD was found between drug- and placebo-treated cats. This experiment does not negate the possibility of *in vivo*

TABLE III. Effect of Benzodiazepines on cAMP Phosphodiesterase Activity in Different Regions of Cat Brain.[a]

Name	% Inhibition		
	Temporal cortex	Hippo-campus	Cere-bellum
Chlordiazepoxide HCl	29**	15**	18*
Diazepam	55***	44***	42***
N-Demethyldiazepam	34***	22**	15*
N-Methyloxazepam	33**	19***	27**
Oxazepam	15	11*	11

[a] Substrate cAMP 5μM. Drug concentration 50 μM.
* $P < .05$.
** $P < .01$.
*** $P < .001$.

inhibition of CNS cAMP-PD because sample preparation prior to enzyme assay would allow ample time for the benzodiazepine to become dissociated from the enzyme. A true *in vivo* expression of inhibition of cyclic nucleotide PD might be reflected by changes in intracellular levels of cAMP and cGMP in the different regions of the CNS.

Discussion. Two previous studies have described the regional distribution of cAMP-PD in the brain of the rabbit (9) and rat (10). Both studies agree with ours in finding high activity in the cortex and low activity in the pons and spinal cord. Our data also show that a phosphodiesterase with a high specific activity for cGMP resides within the CNS of the cat.

Electrophysiological studies provide evidence for a selective action of chloridazepoxide HCl on the hippocampus of the immobilized cat (3). We therefore sought for a biochemical correlate of this physiological event. The data in Table II indicate that medazepam HCl caused significant inhibition of PD activity throughout the brain. The levels of significance suggest that there is little, if any, evidence for a selective action of medazepam HCl on any particular region of the brain.

It should be noted that active members of the benzodiazepine series have activity comparable to the most-active known cyclic nucleotide phosphodiesterase inhibitors, papaverine (5) and dipyridamole (5). The benzodiazepines are significantly more active than theophylline, the most widely used phosphodiesterase inhibitor. From the compounds studied, it is difficult to state definite structural requirements for phosphodiesterase inhibitory activity within the benzodiazepine series. Our data suggest that substitution with methyl in the 1-position is necessary, as illustrated by the decreased activity of *N*-demethyldiazepam and oxazepam in comparison with diazepam. Activity resides within both the benzodiazepine and benzodiazepinone radicals because medazepam HCl and diazepam have similar activity. It is also worthy of note that diazepam is more active than its metabolites *N*-demethyldiazepam, *N*-demethyloxazepam, and oxazepam.

Summary. Cyclic AMP and cGMP phosphodiesterase activity was found in the supernatant fraction of 15 freshly dissected regions of the cat central nervous system. Highest activity was found in the cerebral cortex; lesser activity was observed in other regions of the CNS. Diazepam and medazepam HCl were as active as the known cAMP-PD inhibitors papaverine and dipyridamole in cat cortex, rat brain, and mouse neuroblastoma cells. The diazepam metabolites *N*-demethyldiazepam, *N*-methyloxazepam, and oxazepam showed less inhibition of PD than the parent compound. No evidence was found for a regionally specific biochemical action of the benzodiazepines involving intracellular cAMP or cGMP.

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