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Depression of Some Drug-Induced *in Vivo* Changes of Cerebellar Guanosine 3',5'-Monophosphate by Control of Motor and Respiratory Responses

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SUMMARY

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Paralysis of rats with *d*-tubocurarine and maintanence of normal arterial pH, CO_2 and O_2 tensions reduced cerebellar cGMP to values only one fourth to one third that of spontaneously roving animals. Since administration of *d*-tubocurarine intracisternally elevated rather than decreased cerebellar cGMP, the decrease in nucleotide content may be a result of decreased motor behavior and subsequent cerebellar afferent stimulation. In paralyzed rats an increasing mechanical distortion of the chest by increasing tidal volumes raised cerebellar cGMP when arterial gas tensions were held constant. The relative decreases in cerebellar cGMP produced by pentobarbital, ethanol, and halothane in spontaneously active rats were sharply reduced in paralyzed rats. The magnitude of cGMP elevation produced by some drugs (thyrotropin releasing hormone, apomorphine) is also reduced when secondary motor and respiratory effects of these drugs are prevented, whereas the increase produced by harmaline is not altered. Systematic variations of arterial CO_2 and O_2 tensions revealed a negative correlation between the log cGMP content with arterial CO_2 tension and a positive correlation of the log arterial O_2 tension with cerebellar cGMP content in hypercarbic rats. It is concluded that a significant portion of the drug-induced changes in cerebellar cGMP content produced *in vivo* may be secondary to altered motor and/or respiratory actions of these drugs.

INTRODUCTION

In the last several years many investigators have explored the effects of drugs that alter central transmitter mechanisms on the systems responsible for generating and destroying cAMP[1] and cGMP. These studies have attempted to study the importance of transmitter systems that may determine cyclic nucleotide levels both *in vitro,* using cell fractions, homogenates, or tissue slices, and *in vivo* (for review, see 1). The intriguing aspect of the *in vivo* studies has been the dramatic changes in the cerebellar

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[1] The abbreivations used are: cAMP, adenosine 3',5'-cyclic monophosphate; cGMP, guanosine 3',5'-cyclic monophosphate; TRH, thyrotropin releasing hormone; GTP, guanosine 5'-triphosphate.

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cGMP content—far greater than changes in any other brain region—after administration of drugs that perturb central nervous system function. Thus, central nervous system depressants as diverse as diazepam (2), pentobarbital (3), ethanol (4), halothane (5 and Mueller *et al.*, unpublished observation) and haloperidol (6) all sharply reduce cerebellar cGMP. Similarly, drugs that produce an increased behavioral activity such as apomorphine (6), amphetamine (7), and methylphenidate (Breese *et al.*, unpublished observations), or that induce tremors such as TRH (8), harmaline (2), and oxotremorine (9) are associated with an elevation in cerebellar cGMP. In many of these studies a correlation between the altered behavioral or motor activity and the change in cerebellar cGMP has been suggested; the question of whether these cyclic nucleotide changes contribute to the development of the altered behavior or are only the result of such behavioral changes has not been resolved.

Because it is known that many central nervous system depressant drugs that reduce cerebellar cGMP also depress respiration at high doses, and that some drugs such as apomorphine (10) and TRH (11) increase cerebellar cGMP as well as stimulate ventilation, changes in respiratory control and altered carbon dioxide and/or oxygen tensions may also alter cGMP values. The present study was designed to determine if paralysis and controlled ventilation altered the drug-induced changes in cerebellar cGMP. Our results suggest that in the spontaneously roving rat peripheral sensory inputs to the cerebellum may be responsible for maintaining a major portion of the resting cerebellar cGMP content, and that changes in peripheral motor activity and/or respiratory efficiency may be responsible for a large portion of the alterations in cerebellar cGMP produced by some drugs.

METHODS

Animals preparation. Male Sprague-Dawley rats, 150–180 g, were obtained from Charles River and were housed in a light time cycle (600–1800) facility for at least four days. Animals were prepared for controlled ventilation by placing them in an ether-saturated atmosphere until their righting reflex was lost. Then a small incision was made to expose the trachea to permit visual verification or oral-endotracheal tube placement (a 14 g Angiocath, Deseret Pharmaceutical Co., Inc.). A second small incision was made to expose one femoral artery, and a 23 ga needle was placed in a tail vein. Animals were then paralyzed by administration of 1.5–3.0 mg/kg *d*-tubocurarine i.v., the trachea was intubated, and ventilation controlled using a Harvard rodent respirator (60/min). Both incisions were swabbed lightly with a dilute lidocaine solution. About four to five minutes elapsed from the start of ether anesthesia until respirations were controlled. Thirteen minutes after endotracheal intubation 0.6 ml of blood was slowly withdrawn over 60–90 sec into a 1 ml syringe rinsed in heparin (1000 u/ml). Arterial pH, carbon dioxide ($PaCO_2$) and oxygen (PaO_2) tension were measured with an Instrumentation Laboratory, Inc. 113 blood gas analyzer. Fifteen minutes after the start of controlled ventilation the animal was quickly disconnected from the ventilator and microwave exposure begun within 3–4 sec using a plastic holder and the head exposed to microwave irradiation (Gerling-Moore, Metabostat, 3.5 kW for 2.0–2.2 sec). The head was severed from the body and placed in ice water for 3–5 min before removal and dissection of the brain on crushed ice. All spontaneously active and paralyzed groups used for any drug treatment and their control groups were treated and used the same day.

Cyclic nucleotide assay. Brain regions were homogenized in 10–50 volumes of 0.4 N $HClO_4$ using a polytron (Brinkman Instruments). After centrifugation at 22,000 × *g* for 15 min, the supernatant was removed and stored frozen until analysis. No deterioration of cAMP or cGMP was noted during storage of up to two months. The pellets were dissolved in 1 N NaOH and aliquots removed for determination of protein by the method of Lowry *et al.* (12). For analysis of the cyclic nucleotides the supernatants were adjusted to pH 6.1–6.2 with 2 M Tris and after appropriate dilution ali-

quots were removed for determination of cAMP or cGMP, using the radioimmunoassay of Steiner *et al.* (13). Selected samples from some experiments were processed via the column purification methods of Mao and Guidotti (14), using the protein binding assay for cAMP as specified, or radioimmunoassay as above. In all cases absolute values agreed within 20% (Breese *et al.*, unpublished observations). Because highly complex column purification methods failed to yield any gain in sensitivity, cross-reactivity of various nucleotides, or precision, the simpler direct radioimmunoassay of diluted samples was used routinely.

Drugs. Harmaline was purchased from Sigma Chemical Co.; apomorphine from Merck and Co., Inc.; halothane from Ayerst Laboratories; and sodium pentobarbital and *d*-tubocurarine from Abbott Laboratories. Thyrotropin-releasing hormone was a gift of Abbott Laboratories. All animals were ventilated with room air, except where stated. When carbon dioxide or oxygen was added to inspired gases, Matheson certified gases were employed.

Assay of cerebellar guanylate cyclase. The procedure of Murad *et al.* (15) was modified as follows. The cerebellum (*ca.* 250 mg) from a decapitated rat that had not been exposed to microwave radiation was rapidly rinsed in a cold sucrose solution at 4°, blotted, and weighed. The tissue was homogenized in 3 ml of 0.25 M sucrose–0.005 M Tris-HCl buffer, pH 8.0 at 4° in a hand-held, all-glass homogenizer. The whole homogenate was diluted by 1:8 in the same buffer for use in the assay.

The composition of the final reaction mixtures was 0.05 M Tris-HCl; 0.01 M theophylline; 0.15 M creatine phosphate; 40 μg creatine phosphokinase (*ca.* 140 units/mg); 0.001 M GTP; 0.004 M $MgCl_2$; and enzyme in a total volume of 0.2 ml. The enzyme preparation (0.05 ml) was preincubated for 10 min at 37° with 0.05 ml Tris-HCl buffer at a pH which was varied from 6.6 to 9.0, and 0.05 ml of a solution containing the theophylline, creatine phosphate, and creatine phosphokinase. The reaction was initiated by the addition of GTP and $MgCl_2$ in 0.05 ml. Incubations were carried out at 37° for 10 min, and terminated by the addition of 1.8 ml 0.05 M sodium acetate buffer, pH 4.0, followed by heat inactivation at 90° for 3 min. Reaction velocities were linear with respect to amount of homogenate added over the range of dilutions used. Reaction velocity was also linear for as long as 15 min. The reaction mixture pH was found to change significantly during the conditions used from that of the initial buffer. All pH values refer to final reaction pH. The cGMP was measured as described previously (8).

Statistics. Statistical comparisons between control and treatment conditions were made with either Student's *t*-test or Tukey's procedure in an analysis of variance (16). Tests of correlation were performed using either the raw data or log-transformed values.

RESULTS

Drugs which decrease cerebellar cGMP. In order to determine if the decreased cerebellar cGMP associated with the administration of central nervous system depressant drugs might be the consequence of a decrease in motor activity rather than its cause, several depressants were given both to freely moving animals and to animals immobilized by paralysis with *d*-tubocurarine. Treatment of freely mobile animals with graded doses of ethanol produced progressive depression of cerebellar cGMP 15 min later (Fig. 1) without significantly changing cAMP. Animals that were previously paralyzed (see METHODS), however, did not have significantly decreased cerebellar cGMP values at the lower 0.75 g/kg dose, a dose which in unparalyzed rats produced a dramatic 30% ($p < 0.05$) decrease in cGMP content. Nevertheless, the higher 1.5 g/kg dose of ethanol, which decreased cerebellar cGMP of spontaneously moving (or ventilating) rats by 70%, did produce a modest yet significant ($p < 0.05$) 15% decrease in values of paralyzed rats. Despite the marked decrease in the magntidue of ethanol-induced changes in cGMP in spontaneously roving rats, perhaps the more startling observation in this experiment was that the cerebellar cGMP of animals paralyzed for 15 min and ventilated to maintain arterial pH of 7.41 ± 0.03, PaO_2 = 86 ± 3

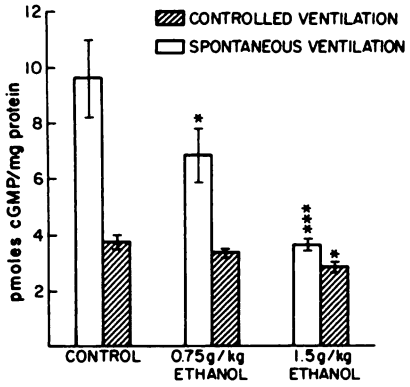

FIG. 1. *Ethanol-induced decrease in cerebellar cGMP*

Animals were either anesthetized, paralyzed and ventilated to maintain normal arterial pH, CO_2 and O_2 tensions, as described in METHODS (controlled ventilation), or allowed normal activity (spontaneous ventilation). All animals received either 20 cc/kg saline i.p. (control) or 0.75 g/kg or 1.5 g/kg i.p. ethanol in saline at volumes comparable to control rats, 15 min before microwave fixation. Each bar represents the mean ± SEM (brackets) of 5 rats. * $p < 0.05$; *** $p < 0.001$.

and $PaCO_2$ of 40 ± 2 Torr was only 40% of control animals. The cGMP content of brain stem, corpus striatum, or cerebral cortex, and the cAMP content of all areas was not similarly altered by paralysis. For this reason the effect of various portions of the experimental protocol short of paralysis and tracheal intubation was examined for the effect of these interventions on cerebellar cGMP. As seen in Figure 2 the ether exposure, the skin incisions, lidocaine, and intravenous saline volume administration produced a significant decrease of cGMP only at 2 min, the interval before the righting reflex had been restored. The relative change in cerebellar cGMP produced by ethanol in these animals that received all manipulations short of paralysis and intubation was not significantly different from that seen in spontaneously roving control rats.

Although *d*-tubocurarine does not cross the blood-brain barrier in appreciable amounts (17), we examined the effects of central *d*-tubocurarine administration on cerebellar cGMP content to make certain that the decrease in cGMP observed in paralyzed rats was not an effect of central nervous system entry of the drug. Preliminary experiments (Mailman *et al.*, unpublished observations) suggested that the nonspecific 20–50% elevation of cerebellar cGMP that occurs with intracisternal administration of 5 μl saline could be prevented by incising the skin under light ether anesthesia 45 min, and administering 20 mg/kg morphine sulfate 30 min before intracisternal injection. This pretreatment protocol was used, and animals were killed by microwave fixation of the brain 15 min after intracisternal administration of 15 μg *d*-tubocurarine. This dose produced a cerebellar cGMP content of 19.8 ± 2.4 pmoles cGMP/mg protein, over threefold higher than rats given a comparable volume of saline (6.2 ± 0.8 pmoles cGMP/mg protein). Rats that received the ether exposure but no morphine had a cerebellar cGMP of 7.5 ± 0.6 pmoles cGMP/mg protein 30 min later. The animals that received i.c. *d*-tubocurarine evidenced continuous running movements, with convulsions occurring in some.

Like effects of ethanol, the administration of sodium pentobarbital 25 mg/kg to

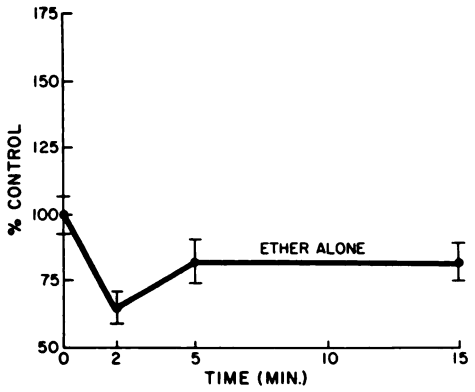

FIG. 2. *Effect of light ether anesthesia, incision, topical lidocaine and intravenous saline injection*

Animals were handled as described in METHODS, except saline was injected instead of *d*-tubocurarine. Ether administration was then stopped (zero time) and animals were then allowed to recover until death by microwave exposure at the indicated time intervals after discontinuing ether. The 0 time values refer to values in animals killed on the same day but not anesthetized, etc. Each value represents the mean ± SEM (brackets) of 5–6 control rats. Only the values 2 min after ether are significantly different from control rat values ($p < 0.05$).

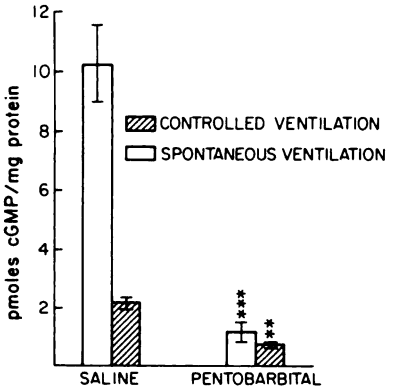

FIG. 3. *Effect of sodium pentobarbital on cerebellar cGMP*

Rats were anesthetized, paralyzed, and ventilated to maintain normal arterial pH, CO_2 and O_2 tensions as described in METHODS (controlled ventilation) or allowed normal activity (spontaneous ventilation). All animals received either 2.0 ml saline i.p. (saline) or 25 mg/kg sodium pentobarbital 15 min before microwave fixation. Each bar represents the mean ± SEM (brackets) or 6 rats. ** $p < 0.01$; *** $p < 0.001$, compared to saline treated rats.

freely moving rats significantly decreases cerebellar cGMP 15 min later (Fig. 3). In paralyzed animals the relative change was much less, though still significant ($p < 0.05$). Administration of halothane (1.5% in air) produced a loss of the response to tail pinch in spontaneously breathing rats 14 ± 1.5 min later (Fig. 4). This endpoint of anesthetic effect was associated with a 70% decrease in cerebellar cGMP. When other rats were paralyzed and ventilation maintained at normal levels during 15 min exposure to 0.7, 1.4, or 2.1% halothane in air, the decrease in cerebellar cGMP, while still significant, was nonetheless sharply reduced.

Drugs that elevate cerebellar cGMP. Administration of TRH produces a dose-dependent elevation of cerebellar cGMP (8). As seen in Figure 5, however, the effect of a dose of TRH that increased cerebellar cGMP threefold in the spontaneously moving rat produced a much smaller, though significant, 50% elevation. The relative increase in cerebellar cGMP produced in animals that received all manipulations short of paralysis and tracheal intubation were not significantly different from that of spontaneously roving animals.

With apomorphine administration (3 mg/kg) a motor activity increase was observed (18). This was accompanied by a threefold increase in cerebellar cGMP (Fig. 5). In paralyzed rats, however, the increase was more modest (+50%), yet once again still statistically significant.

In animals given harmaline (6.4 mg/kg) the relative increase in cerebellar cGMP 15 min later was similar in both freely moving and in paralyzed rats (Fig. 5).

Alterations in respiratory gas tensions and cerebellar cGMP. Ventilation of rats at various tidal volumes to produce a spectrum of arterial $PaCO_2$ values revealed that cerebellar cGMP varied inversely with $PaCO_2$ (19). The correlation coefficient of $PaCO_2$ and log transformed values of cerebellar cGMP pmoles/mg protein was higher than when nontransformed cGMP values were used (Fig. 6). Both r values were statistically significant. A similar correlation of cGMP with $PaCO_2$ was noted in the brain stem; however no significant correlation was noted in the caudate nucleus or cerebral cortex. Values of $PaCO_2$ did not correlate with cAMP in any brain region.

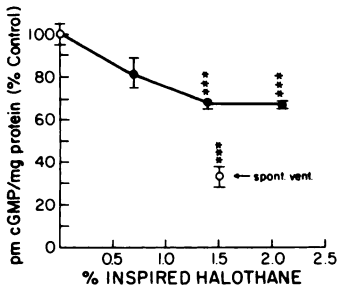

FIG. 4. *Effect of halothane exposure on cerebellar cGMP*

Rats were either anesthetized, paralyzed, and ventilated to maintain normal arterial pH, CO_2 and O_2 tensions as described in METHODS (●), or were permitted spontaneous ventilation (○). Control values for cerebellar cGMP in spontaneous ventilated rats were 13.5 ± 2.3 pmoles cGMP/mg protein; control values of ventilated rats were 4.60 ± 0.23 pmoles cGMP/mg protein. Spontaneously active rats were exposed with 1.5% halothane in air until their response to tail pinch was lost at 14 ± 1.3 min later, when they were killed by microwave exposure. All controlled ventilation rats (●) were killed by microwave exposure 15 min after the start of halothane exposure. *** $p < 0.001$.

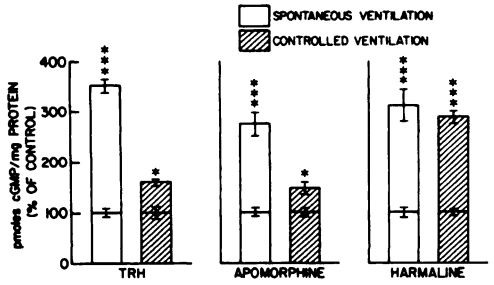

FIG. 5. *Effect of TRH, apomorphine, and harmaline on cerebellar cGMP in paralyzed and spontaneously active rats*

See Fig. 3 for group designations. TRH-treated rats received 10 mg/kg (in 5 cc saline/kg) i.v. 15 min before microwave fixation. Control rats received comparable volumes of saline. Control spontaneous ventilation rats had cerebellar cGMP values of 5.6 ± 0.4 pmoles/mg protein; controlled ventilation rats had 1.75 ± 0.24 pmoles/mg protein. Apomorphine-treated rats received 3 mg/kg i.p. apomorphine hydrochloride in 2.0 cc saline/kg 1% ascorbic acid i.p. 15 min before microwave fixation. Control rats received comparable volumes of the ascorbic acid–saline vehicle. Harmaline-treated rats received 6.4 mg/kg i.v. in saline (2.5 ml/kg) 15 min before microwave fixation. Control rats received comparable volumes of saline. Control values of cerebellar cGMP in spontaneous ventilation rats was 12.4 ± 1.7 pmoles/mg protein, and in controlled ventilation rats, 4.4 ± 0.4 pmoles/mg protein. Each bar represents the mean ± SEM (brackets) of 6–7 rats. * $p < 0.05$, *** $p < 0.001$ relative to similarly ventilated rats given saline (100% of control group). Only in the harmaline experiments was the relative effect in spontaneous ventilation and controlled ventilation groups not significantly different ($p > 0.05$).

Since altering $PaCO_2$ by changing the tidal volume produces a concomitant change in excursion of the thorax and abdominal structures, the influence of *in vivo* $PaCO_2$ on cerebellar cGMP was investigated under conditions in which no mechanical changes occurred. To accomplish this one group of rats was hyperventilated with air to produce a mean $PaCO_2$ of 21 ± 1.3 Torr, and another group of rats was ventilated with a similar frequency and tidal volume, but a mixture of 5% carbon dioxide, 20% oxygen, and 75% nitrogen was used (Fig. 7). The $PaCO_2$ produced in this second group (39 ± 1.2 Torr) was not significantly different from that in animals that were paralyzed and ventilated at a lower tidal volume with air to maintain a normal $PaCO_2$ (40 ± 4.5 Torr). In the cerebellum only a portion of the increase in cGMP produced by hyperventilation was reduced by elevation of $PaCO_2$ without change in respiratory excur-

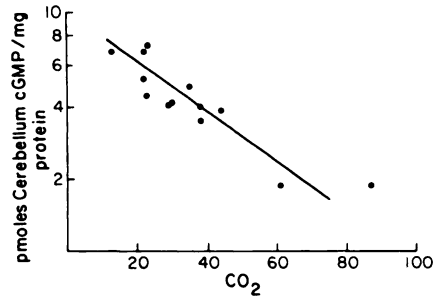

FIG. 6. *Effect of variation of arterial carbon dioxide tension on cerebellar cGMP*

Animals were anesthetized, paralyzed, and ventilated with air as described in METHODS. Each point represents a single animal. Correlation coefficient of log cGMP pmoles/mg protein was 0.764.

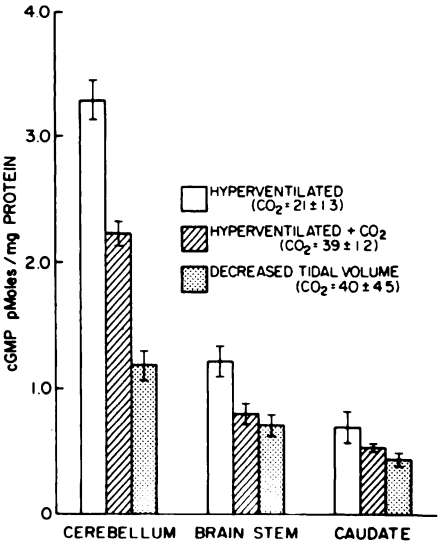

FIG. 7. *Comparative effect of a decrease in tidal volume and the addition of CO_2 to decrease regional brain cGMP in hyperventilated rats*

Rats were anesthetized, paralyzed and ventilated with air (open and stippled columns) or a mixture of 5% carbon dioxide, 20% oxygen and 75% nitrogen (cross-hatched bars). The large tidal volume used in the animals of both the open and cross-hatched bars was identical, and produced a $PaCO_2$ of 21 ± 1.3 in the animals given room air (unfilled bars). Arterial oxygen tensions were 90 ± 1.9 in animals hyperventilated in room air, 101 ± 1.9 in hypoventilated rats given supplemental CO_2 and 76 ± 6.8 in rats with decreased tidal volume. The height of each bar represents the mean ± SEM (brackets) of six animals.

sion, whereas in the brain stem the change was abolished. Thus some of the elevation in cerebellum cGMP produced by hyperventilation may have been the result of augmented thoracic movement.

Previous studies demonstrated that increasing the inspired oxygen tension in the hyperventilated rat lowered cerebellar cGMP, whereas a slight elevation was observed in the lower cGMP levels of hypoventilated rats (19). Those studies revealed no significant correlation of cGMP in any brain region with PaO_2 when PaO_2 was varied in hyperventilated or normal ventilated animals by the addition of oxygen to the inspired air. As seen in Figure 8, however, in the presence of a $PaCO_2$ of 71 ± 3 Torr, the cerebellar cGMP correlated positively with oxygen tension. The correlation coefficient for logarithmically transformed PaO_2 values was much higher with cerebellar cGMP than that observed with the untransformed PaO_2 values, though both correlation coefficients were statistically significant.

To determine of CO_2 or O_2 might modulate cerebellar cGMP levels directly by increasing cerebellar guanylate cyclase activity, the effect of changes of these gas tensions was examined *in vitro*. Incubation of reaction vessels in atmospheres of 100% nitrogen or 100% oxygen did not produce detectable changes in *in vitro* enzyme activity in air. Increasing CO_2 tensions did decrease cyclase activity, but adjusting the pH to 7.4 abolished the CO_2 effect. Figure 9 demonstrates that there was indeed appreciable depression in *in vitro* cerebellar

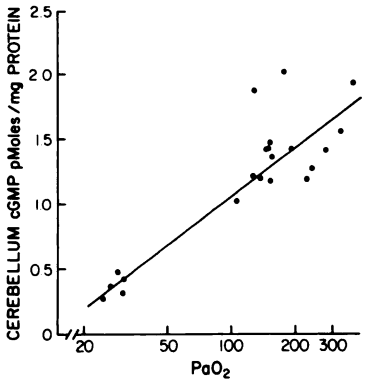

FIG. 8. *Effect of variation of arterial oxygen tension on cerebellar cGMP in hypoventilated rats*
Animals were anesthetized, paralyzed, and hypoventilated ($PaCO_2$ = 71 ± 0.3 mmHg) with various mixtures of oxygen in air. Each point represents the value for one animal. The correlation coefficient of pmoles cGMP/mg protein with log PaO_2 was 0.878.

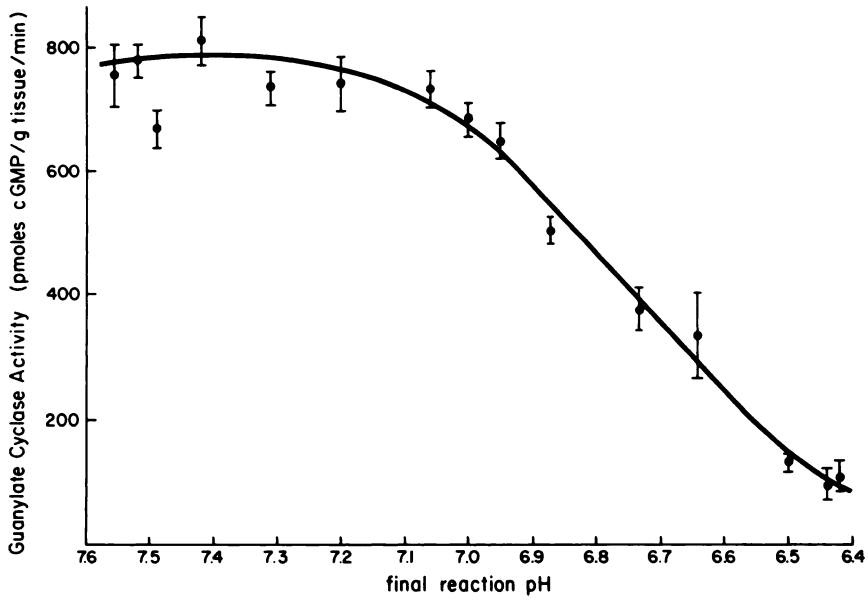

FIG. 9. *Variation of cerebellar guanylate cyclase activity as a function of pH*
Tissue from several untreated rats was removed, and cerebellar homogenates used to measure guanylate cyclase activity as outlined in METHODS. All pH values represent the pH at the end of the incubation, and each point represents the mean ± SEM (brackets) of four replicate fractions.

guanylate cyclase as the pH was decreased but this was clearly seen only when pH fell below 7.1.

DISCUSSION

The present results demonstrate that cerebellar cGMP content is sharply reduced after 15 min of paralysis induced with *d*-tubocurarine and maintanence of normal blood gas tensions and pH. The spinal cord conducts information to the cerebellum; the information integrated in this region permits position perception and smooth coordinated movement. These impulses are largely transmitted to the cerebellum by changes in frequency or perhaps pattern of stimulation of mossy and climbing fibers (20). The work of Ishikawa *et al.* (21) and Leicht *et al.* (22) have demonstrated that both cutaneous and tendon receptor stimulation are associated with an increase in activity of both afferent fiber types. Because the activity pattern produced by *d*-tubocurarine, when administered iontophoretically to cell groups from which climbing fibers such as the dorsal accessory olive arise, is only a frequency discharge similar to that of harmaline (23), one might expect an elevation of cerebellar cGMP if some of the *d*-tubocurarine had penetrated the central nervous system. In fact, intracisternal administration of *d*-tubocurarine did markedly elevate cerebellar cGMP, coincident with the appearance of running behavior and some convulsive activity. These results, and the report that *d*-tubocurarine does not cross the blood-brain barrier (17), make it unlikely that central effects of the drug rather than the ensuing paralysis caused the decrease in cerebellar cGMP observed after paralysis with *d*-tubocurarine. After paralysis, the normal afferent stimuli intensity is presumably reduced because of a decreased proprioceptor input from muscles and tendons, as well as the absence of tactile stimulation during exploring behavior, etc. The importance of peripheral afferent input on cerebellar cGMP is highlighted by the subsequent experiments in which changes in tidal volume of ventilation produced different values of cerebellar cGMP, even when CO_2 tensions were held constant by the addition of CO_2 to the group with greater tidal volumes. The increased tidal volumes may have raised cerebellar cGMP by the greater distortion of thoracic and abdominal contents and chest wall musculature.

The administration of central nervous system depressants used in this study—ethanol, pentobarbital, and halothane—produced a significant decrease in cerebellar cGMP in paralyzed rats when high enough doses were employed. However, when changes in motor activity are controlled by total paralysis the magnitude of the decline in cGMP seems to be much less striking than that observed in spontaneously moving rats. Thus a majority of the decline in cerebellar cGMP observed in spontaneously moving animals may be due to the decreased motor activity rather than to direct cerebellar or remote central nervous system induced cerebellar depression. Although once cerebellar cGMP values are reduced after *d*-tubocurarine, the relative drug-induced decrease may be less because a minimal limit to the cGMP value exists that cannot be further decreased, 4–8 fold variations in cerebellar cGMP are observed in paralyzed rats when CO_2 or O_2 tensions are altered. Thus, a considerable change in cerebellar cGMP can still be produced in paralyzed rats.

The interpretation of a wealth of data obtained in freely moving animals after drugs or environmental treatments (see INTRODUCTION) must thus be re-evaluated using the knowledge that unrelated changes in motor activity may be responsible for the altered cerebellar cGMP contents. The hazards of extending these studies to more complex drug interaction studies of blockade or potentiation are also evident.

The elevation in cerebellar cGMP produced by both apomorphine and TRH was sharply reduced in paralyzed animals. Apomorphine has been proposed as a dopaminergic receptor agonist and has been postulated to elevate cerebellar cGMP by indirectly increasing mossy fiber activation (6, 24). Thyrotropin releasing hormone has been shown to arouse animals from pentobarbital narcosis by a mechanism that can be antagonized by atropine (25). Thus the mechanism of action of TRH that elevates cerebellar cGMP may be similar to that of oxotremorine (9). Because apomorphine

and TRH are known to increase spontaneous motor activity in rats and mice (8, 26), both agents probably also increase peripheral cerebellar afferent stimulation. The much more modest (although still significant) increases in cerebellar cGMP found in paralyzed rats suggests that a portion of the increase in cerebellar cGMP observed with these drugs in spontaneously roving animals is secondary to this increased motor activity. Experiments designed to examine the transmitter mechanisms responsible for these changes *in vivo* would thus be best done when changes in motor activity could be controlled or at least quantitatively measured.

Harmaline is known to activate cerebellar neurones by stimulating the climbing fibers that originate in certain brain stem nuclei (27). Thus compared to apomorphine or TRH, which presumably activate the cerebellar cells by polysynaptic extracerebellar central nervous system mechanisms, harmaline acts more proximally on or near an afferent climbing fiber cell body (18, 23). Interestingly, the absence or presence of paralysis did not affect the relative increase in cerebellar cGMP observed after this tremorigenic drug. This finding reinforces our interpretation of the other drug effects reported in this paper, namely that many of the observed changes in cyclic GMP are secondary to alterations in motor activity. Indeed, Rubin and Ferrendelli (28) have recently demonstrated that simply shaking an animal can elevate cerebellar cGMP.

Changes in respiration also appear to be an important determinant of *in vivo* cerebellar cGMP content. Thus increases in $PaCO_2$ tensions produced either by decreasing the tidal volume of ventilation, or by adding CO_2 to the inspired air, produce a progressive reduction in cerebellar cGMP. Because haloperidol does not alter cerebellar cGMP in paralyzed rats (29), it seems unlikely that hyperventilation increases cerebellar cGMP by dopaminergic system activation. Apart from some indirect neuronal mechanisms, this CO_2 dependent decrease in cGMP content also could be the result of either lowered pH *per se* in cells of the cerebellum, or secondary to changes in central nervous system blood flow, which is directly related to $PaCO_2$ tensions (30).

An increase in blood $PaCO_2$ is accompanied by a progressive decrease in central nervous system pH (31). Such an intracellular acidosis might decrease guanylate cyclase activation and thus cGMP content. Indeed, Nakazawa and Sano observed a linear sharp decrease in whole rat brain guanylate cyclase activity when pH level fell below 7.8 (32). Our results with whole homogenates of cerebellum, the region which appears most sensitive to CO_2 exposure, indicate that a much lower pH is necessary. Although cerebellar guanylate cyclase activity is pH sensitive *in vitro,* the deviation from full activity at pH 7.4 does not begin until the final pH of the reaction mixture if below 7.0. This level, even measured in blood, is actually achieved only with the most extreme variations of $PaCO_2$ produced in this study. Thus it would seem that cellular acidosis, which presumably is less profound than that produced in blood by CO_2 accumulation, would not explain the decrease observed in cerebellar cGMP with modest increases in $PaCO_2$. Messeter and Siesjö (33) have demonstrated that $PaCO_2$ elevation similar to that achieved in this study for as short a time as 15 min decreases whole brain glutamate, and Van Leuven *et al.* (34) demonstrated an increase in gamma aminobutyric acid (GABA) with respiratory acidosis. Glutamate has been proposed to be an excitatory transmitter to cerebellar Purkyñje cells (35), and since analogues of glutamate elevate cerebellar cGMP *in vitro* (36), a loss of glutamate availability may produce a decrease in cerebellar cGMP. Similarly, locally applied muscimol, an analogue of GABA, decreases cerebellar cGMP when administered directly into the cerebellum *in vivo* (24). Thus changes in either of these two putative transmitter systems may also contribute to the observed decrease in cGMP with increase in CO_2 tension.

A second possible mechanism by which an increase in $PaCO_2$ may decrease cerebellar cGMP is suggested by the report of Sjölund *et al.* (18). These investigators reported that serotoninergic nerve terminals in the dorsal accessory olive of the rat may tonically inhibit climbing fiber activity. Thus harmaline has been postulated to increase climbing fiber activity as a result of

presynaptic inhibition of these serotonin containing nerve terminals. Davis and Carlsson (37) have previously demonstrated that serotonin synthesis *in vivo* is controlled by tissue oxygen tension, and since an increase in $PaCO_2$ tension increases blood flow to the brain (29), venous (and presumably cellular) oxygen tension is increased (38). Such an elevation of cellular oxygen tension could thus decrease climbing fiber activation by increasing serotonin availability. Thus an increase in $PaCO_2$ may decrease cerebellar cGMP content by increasing the degree of serotoninergic inhibition of this cerebellar afferent input.

Changes in arterial oxygen tension did not correlate positively with cerebellar cGMP when $PaCO_2$ was held normal or was decreased. Since systematic changes in oxygen content in hypoventilated rats, with an elevated $PaCO_2$, did reveal a significant positive correlation with PaO_2, it is possible that near maximal cerebral perfusion is required in order to produce significant changes in cellular oxygen tension. Since *in vitro* guanylate cyclase activity was not altered by exposure to 100% O_2 or N_2 atmosphere, this effect may require an intact cell architecture or other central nervous system inputs. It appears that the increased oxygen tension does not elevate cerebellar guanylate cyclase activity by simply increasing superoxide or peroxide availability as has been observed in studies in the liver (15). However, in *in vitro* conditions selected, hydroxyl radical availability may be maintained by anaerobic processes at an already maximal level; thus an effect would only be visible in unbroken cells.

When the effects of drugs that decrease or increase respiration are examined for their effects on cerebellar cGMP, any observed changes may be secondary to the associated changes in respiratory function as well as motor activity.

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