Biochemical Pharmacology, Vol. 23, pp. 1817–1825. Pergamon Press, 1974. Printed in Great Britain.

EFFECTS OF Δ^9-TETRAHYDROCANNABINOL ON THE LEVELS OF CYCLIC ADENOSINE 3′,5′-MONOPHOSPHATE IN MOUSE BRAIN*

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(*Received* 16 *August* 1973; *accepted* 16 *November* 1973)

Abstract—The effects of intraperitoneal injections of various doses of Δ^9-tetrahydrocannabinol (Δ^9-THC) on brain levels of cyclic adenosine 3′,5′-monophosphate (cyclic AMP) have been studied in mice. Doses of Δ^9-THC in the range of 0·1 to 1·0 mg/kg cause a 50–160 per cent elevation of cyclic AMP levels compared to controls ($P < 0.0005$), while doses of Δ^9-THC in the range of 2·0 to 10·0 mg/kg cause a 30–60 per cent depression of cyclic AMP levels ($P = 0.025$ to 0·0005). This pattern was obtained in whole brain, as well as in dissected samples of cortex, cerebellum and medulla. This over-all biphasic effect of THC on cyclic AMP levels correlates with known changes in biogenic amines, temperature regulation, and behavior caused by this drug.

NUMEROUS investigators have demonstrated that tetrahydrocannabinol (THC) and at least one of its metabolites, 11-OH-THC, are the major compounds responsible for the psychomimetic action of marihuana in mammalian systems.[1–14] Although the molecular mechanism of THC action is still unknown, evidence has accumulated suggesting that the effects of *Cannabis* may be mediated, at least in part, by alterations in biogenic amines. Moderate doses of THC or *Cannabis* extracts in rodents have been reported to produce an elevation in brain levels of serotonin[15–19] and a depression of norepinephrine,[16,17,20] 5-hydroxyindole acetic acid and normetaephrine.[21] In view of the importance of these biogenic amines as neurohumors affecting behavior and mood, it seems reasonable to speculate that these changes in brain amines are in some way involved in the behavioral effects of marihuana.

Some biogenic amines are now thought to transmit their effects via an activation of the membrane-associated enzyme adenyl cyclase, resulting in the synthesis of cyclic adenosine 3′,5′-monophosphate (cyclic AMP).[22–27] This hypothesis is based mainly on experiments in which brain slices have been incubated with norepinephrine and were subsequently found to contain increased levels of cyclic AMP, although some cell-free preparations have also been reported where adenyl cyclase activity can be stimulated by catecholamines.[28] Furthermore, in some experimental situations it has been possible to demonstrate that changes in neural discharge rates produced by norepinephrine can be mimicked by direct application of cyclic AMP,[29] thus directly implicating cyclic AMP as a mediator of the effects of biogenic amines on nerve cells. Further support for this conclusion comes from studies which have shown that administration of dibutyryl-cyclic AMP directly into the brain causes a

* Supported in part by Grant 5 RO1 DA00116 (formerly MH 21003) from the National Institute of Mental Health.

variety of behavioral changes, including increased locomotor activity, catatonia and convulsions.[30]

From these types of data it appears that cyclic AMP may be intimately involved in mediating the effects of brain amines on behavior. In view of the reported changes in both biogenic amines and behavior induced by the administration of active ingredients of marihuana such as Δ^9-THC, it seemed appropriate to attempt to determine whether some of the effects of this drug are also mediated by changes in cyclic AMP. The present studies show that significant alterations in brain levels of cyclic AMP are induced by administration of THC to mice. Low doses of THC cause an increase in cyclic AMP levels, while at high doses of THC, the levels of cyclic AMP are depressed. This over-all biphasic effect correlates with reported changes in both biogenic amines and behavior patterns.

MATERIALS AND METHODS

Materials. 1-(−)-Trans-Δ^9-tetrahydrocannabinol (THC) was kindly supplied by Dr. M. Braude at the Center for Studies of Narcotic and Drug Abuse, National Institute of Mental Health. ^{3}H-Δ^9-THC (109 μCi/mg), ^{3}H-cyclic adenosine 3′,5′-monophosphate (24·1 Ci/m-mole) and ^{14}C-ATP (50 mCi/m-mole) were obtained from New England Nuclear Corp.

Injection of animals and extraction of cyclic AMP. CF1-Carworth, male 30- to 45-g mice were used throughout the investigation. They were housed three to five per cage at 24°, photoperiod 16 hr on with food and water *ad lib.* Mice were randomly chosen, weighed and injected intraperitoneally between 9:00 and 12:00 a.m. with THC solutions made up in 0·2 ml of 0·15 M NaCl–1% Tween-80. Experimental animals received THC doses in the range of 0·1 to 50 mg/kg, while control animals received injections of vehicle only. Thirty min after injection the mice were sacrificed by 30 sec of microwave irradiation as described by Schmidt *et al.*[31,32] The animals were decapitated, the heads chilled and the brain was dissected into the medulla oblongata, cerebellum, hypothalamus and remainder (labeled cortex) as described in Glowinski and Iversen.[33]

Each brain region was homogenized in 1–3 ml of 10% trichloroacetic acid with ten strokes of a glass Teflon homogenizer at 7000 rev/min. The resulting homogenate was centrifuged at 10,000 *g* for 20 min and the supernatant collected. The homogenate pellet was extracted with 5 vol. of 20% ethanol in ether prior to a modified Koch–Putnam biuret protein determination[34] using 1:3 proportion of 1 N sodium hydroxide to biuret reagent. Duplicate aliquots of the supernatant from each brain region (0·1 to 0·3 ml) were extracted four times with 20 vol. of ether (saturated with 1·0 N HCl) to remove the TCA prior to the assay of cyclic AMP. Extraction was performed by vortexing for 30 sec in 12 ml acid-washed conical tubes. The extracted aqueous aliquot was dried in a vacuum desicator overnight and resuspended in 50 mM sodium acetate buffer at the time of the assay of cyclic AMP. Tracer ^{3}H-cyclic AMP added to sample brain regions and carried through the procedure showed no counts soluble in the ether phases and a cyclic AMP recovery of approximately 90 per cent.

Protein-binding assay for cyclic AMP. The cyclic AMP-dependent protein kinase was isolated from bovine forelimb muscle employing the procedure of Miyamoto *et al.*[35] through the DEAE-cellulose chromatography step. Cyclic AMP was assayed

according to the protein-binding method of Gilman.[36] Briefly, a standard curve was generated by incubating various concentrations of unlabeled cyclic AMP (0–500 nM) with 16·6 nM ^{3}H-cyclic AMP, 1 μg protein kinase binding protein (peak I from DEAE-cellulose column), 10 μl of inhibitor as isolated by Appleman *et al.*,[37] and 50 mM sodium acetate, pH 4·0, in a volume of 0·1 ml for 2 hr at 4°. Concommitantly, unknowns were incubated with all of the preceding components with the exception of unlabeled cyclic AMP. After 2 hr, the contents of each tube were filtered through a multiple membrane filtering apparatus, and the filters dried and counted in toluene scintillation mixture at 32 per cent efficiency. An IBM 360 computer was employed to generate a regression equation by the method of least squares for the standard curve, and the unknown samples were solved employing regression.

Assay of adenyl cyclase and phosphodiesterase. Adenyl cyclase activity was measured by a modification of the method of Drummond and Duncan.[38] After sacrifice by either decapitation or microwave irradiation, the mouse brains were homogenized in 5 vol. of cold 10 mM Tris–HCl, pH 7·5. The total volume of the assay mixture was 150 μl and contained the following: 40 mM Tris–HCl (pH 7·5), 8 mM theophylline, 15 mM $MgSO_4$, 20 mM phosphoenolpyruvate, 2 mM cyclic AMP, 5·5 mM KCl, 130 μg/ml of pyruvate kinase, 0·4 mM ^{14}C-ATP (16 mCi/m-mole) and 30 μg (protein) of brain homogenate. The reaction was initiated by addition of homogenate and the tubes were incubated for 15 min at 37°. The reaction was terminated by boiling in a water bath for 4 min. The tubes were centrifuged at 2000 *g* for 5 min and 0·1 ml of the supernatant was spotted on Whatman No. 40 chromatography paper and developed by descending technique with *n*-propanol–concentrated ammonia–water (6:3:1) along with standard cyclic AMP. The developed chromatogram was visualized under ultraviolet light and the cyclic AMP regions were cut out and counted in Bray's scintillation mixture.

Phosphodiesterase was assayed by a modification of the method of Brooker *et al.*[39] The entire assay was conducted in a scintillation vial containing 0·15 ml of the following mixture: 8 pmoles ^{3}H-cyclic AMP, 120 mM Tris–HCl, pH 8·0, 0·67 mg/ml of snake venom (King Cobra), 0·67 mg/ml of human serum albumin, 5 mM 2-mercaptoethanol, 2·5 mM EGTA [ethylene-bis(oxethylenenitrilo)tetra-acetic acid], 120 mM $MgCl_2$, 0·12 mM 5′-AMP and 70 μg brain homogenate made up in 10 mM Tris–HCl, pH 8·0. After 10 min of incubation at 37°, 0·8 ml of 50 per cent settled volume of an aqueous slurry of AGl-X2 200–400 mesh anion exchange resin was added to the vials, which were then reincubated for an additional 10 min to absorb the unreacted cyclic AMP. Ten ml of Bray's scintillation mixture was then added and the free radioactive adenosine determined by liquid scintillation counting.

Recovery of injected THC in mouse brain. In order to determine the amount of THC which actually reaches the brain after intraperitoneal injection, recovery studies were performed after intraperitoneal injections of radioactive THC (7·14 mg/kg of ^{3}H-THC, sp. act. = 109 μCi/mg). Thirty min post-injection, the animals were sacrificed by exposure to microwave irradiation for 30 sec, and the brain regions removed and homogenized twice with 5·0-ml portions of ethyl acetate using a Polytron homogenizer for 20 sec at maximum speed. The resulting homogenate was centrifuged at 850 *g* for 5 min and the ethyl acetate phases were pooled and evaporated under nitrogen. The soluble counts were taken up in a small volume of ethanol, spotted on Silica gel G thin-layer chromatography plates along with standards of Δ^9-THC,

and developed with hexane–acetone (3:1). The developed plates were cut in 1 cm^2 blocks and counted in toluene scintillation mixture at 32 per cent efficiency.

RESULTS

Protein-binding assay for cyclic AMP. The preparation of the cyclic AMP binding protein which we obtained was found to have a dissociation constant of 7×10^{-9} M, and at saturation bound 0·6 pmole radioactive cylic AMP/μg of protein. The standard curves obtained were found to have a standard deviation of less than 8 per cent when unlabeled cyclic AMP concentrations were in the range of 0·8 pmole to 5 pmole/0·1 ml of assay mixture. Standard curves with unlabeled cyclic GMP as competitor for radioactive cyclic AMP binding showed this nucleotide to be only 1/30 as effective as unlabeled cyclic AMP in competing for 1 μg of the binding protein. Other nucleotides such as cyclic UMP and cyclic CMP were found to compete even less (1/60) than cyclic GMP.

Fixation of cyclic AMP levels by microwave irradiation. Numerous investigators have reported that when animals are sacrificed by decapitation, endogenous cyclic AMP levels in brain are elevated 2- to 10-fold within 1–3 min.[31,32,36] To prevent such post-mortem changes from occurring, we employed microwave irradiation as a technique for sacrificing animals. As can be seen from the data in Table 1, this technique inactivates both adenyl cyclase and phosphodiesterase, thus preventing the dramatic changes in cyclic AMP levels seen after decapitation.

Recovery of ^{3}H-THC in brain after intraperitoneal injection. Since other investigators have reported relatively poor absorption of THC after intraperitoneal injection,[40] we performed some recovery experiments with radioactive THC in order to determine how much of our injected dose was reaching the brain. Thirty min after intraperitoneal injection of 7·14 mg/kg of ^{3}H-THC, 0·07 per cent of the total radioactivity was recoverable in the brain. Assuming that 80 per cent of the wet brain weight is water, this converts to a concentration of approximately $4·8 \times 10^{-4}$ M. This concentration is within the normal range of THC found in the blood and brain of mammals, as calculated from other studies using various routes of administration and employing behavioral tests as criteria for effective dose.[4,41,42]

Effects of THC on brain levels of cyclic AMP. The effects of injecting various doses of Δ^9-THC on whole brain levels of cyclic AMP are summarized in Fig. 1. As can

TABLE 1. EFFECTS OF DECAPITATION AND MICROWAVE IRRADIATION ON BRAIN LEVELS OF CYCLIC AMP, ADENYL CYCLASE AND PHOSPHODIESTERASE*

Treatment	Cyclic AMP (pmoles/mg brain)	Adenyl cyclase (cpm/mg protein)	Phosphodiesterase (cpm/mg protein)
Decapitation	8·6; 11·6	2056; 2007	12,236; 11,361
Decapitation and immersion in liquid nitrogen	2·6; 4·2		
Microwave irradiation	2·2; 2·8	6; 0	0; 0

* Mice were sacrificed either by decapitation or microwave irradiation, after which brain levels of cyclic AMP, adenyl cyclase and phosphodiesterase were determined as described in the text. Blank values for adenyl cyclase and phosphodiesterase were obtained by boiling samples for 3 min. Each value represents the mean value obtained by assaying one animal in duplicate.

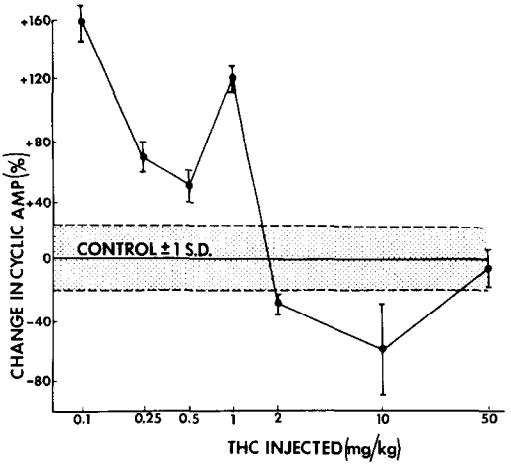

FIG. 1. Change in brain levels of cyclic AMP as a function of injected dose of Δ^9-THC. Data are expressed as percentage change from control values, with the verticle bars representing standard deviations.

be seen, the predominant effect of Δ^9-THC on cyclic AMP levels is an elevation (50–160 per cent) above controls at doses between 0·1 and 1·0 mg/kg, and a depression (30–60 per cent) of cyclic AMP levels at doses between 2·0 and 10 mg/kg. Superimposed on this general trend is a statistically significant variation in cyclic AMP levels in the low dose range which involves a primary stimulation (0·1 mg/kg), then a depression (0·25 to 0·5 mg/kg), followed by a secondary stimulation (1·0 mg/kg). At the highest dose of Δ^9-THC tested (50 mg/kg), cyclic AMP levels were not significantly altered.

Table 2 summarizes the results of statistical analyses performed to determine the significance of the observed trends. As can be seen, the elevations of cyclic AMP levels observed with doses of Δ^9-THC from 0·1 to 1·0 mg/kg, and the depressions of cyclic AMP observed with doses of Δ^9-THC from 2·0 and 10·0 mg/kg are all significant ($P = 0{\cdot}025$ to $0{\cdot}0005$). In this context, it should be noted that measured levels of cyclic AMP in control animals varied some 20 per cent in experiments performed at different times on different batches of animals. Although the source of this variability is unknown (perhaps some daily or circadian rhythm), it emphasizes the need

TABLE 2. STATISTICAL ANALYSIS OF Δ^9-THC EFFECTS ON CYCLIC AMP LEVELS IN MOUSE BRAIN

Dose of Δ^9-THC	N*	Cyclic AMP (pmoles/mg brain protein ± 1 S. D.)	P value†
Control	8	4·9 ± 0·98	
0·1 mg/kg	8	12·6 ± 1·49	0·0005
0·25 mg/kg	8	8·3 ± 0·84	0·0005
0·5 mg/kg	8	7·4 ± 0·84	0·0005
1·0 mg/kg	8	10·8 ± 0·88	0·0005
2·0 mg/kg	3	3·5 ± 0·22	0·025
10·0 mg/kg	8	2·0 ± 0·66	0·0005
50·0 mg/kg	9	4·6 ± 0·54	0·4

* N is the number of animals tested, each assayed in duplicate.

† P values generated from the statistical method for testing the difference between two populations with the population variance unknown but assumed equal, significance for one-sided upper tail hypothesis test.

TABLE 3. DOSE EFFECTS OF Δ^9-THC ON CYCLIC AMP LEVELS IN VARIOUS REGIONS OF MOUSE BRAIN*

Dose	N	Cortex Cyclic AMP (pmoles/mg protein)	P value	Cerebellum Cyclic AMP (pmoles/mg protein)	P value	Hypothalamus Cyclic AMP (pmoles/mg protein)	P value	Medulla Cyclic AMP (pmoles/mg protein)	P value
Control	8	3·7 ± 0·89		7·0 ± 1·30		19·9 ± 3·78		9·4 ± 1·32	
0·1 mg/kg	8	13·4 ± 1·12	0·0005	13·6 ± 1·88	0·0005	15·9 ± 3·99	0·025	14·1 ± 2·86	0·0005
0·25 mg/kg	8	7·9 ± 0·28	0·0005	8·4 ± 2·36	0·08	14·7 ± 1·09	0·0008	12·5 ± 1·32	0·0005
0·5 mg/kg	8	4·0 ± 0·25	0·2	8·3 ± 0·60	0·01	20·3 ± 4·95	0·05	13·6 ± 1·76	0·0005
1·0 mg/kg	8	8·4 ± 0·49	0·0005	18·4 ± 3·08	0·0005	20·0 ± 1·17	0·4	10·4 ± 0·30	0·025
2·0 mg/kg	3	2·5 ± 0·16	0·02	7·6 ± 0·50	0·2	9·4 ± 1·45	0·0005	6·8 ± 0·28	0·007
10·0 mg/kg	8	1·0 ± 0·48	0·0005	4·8 ± 0·58	0·0005	25·8 ± 4·27	0·004	7·2 ± 2·24	0·1
50·0 mg/kg	9	3·5 ± 0·33	0·15	5·6 ± 0·80	0·004	14·6 ± 4·1	0·005	11·7 ± 1·30	0·0008

* Statistical analysis as in Table 2.

to run a new set of control animals with each new experiment, since only in this way can the effects of the drug treatment be reliably determined. Therefore, each individual experiment was performed with several doses and a control group, and the data were normalized to the arithmetic mean of the pooled control groups.

In order to determine whether the observed effects of THC administration on whole brain cyclic AMP levels could be localized to any particular brain areas, cyclic AMP assays were performed on dissected regions of the brain. As is shown in Table 3, the cortex, medulla and cerebellum follow the same general biphasic pattern as that of whole brain, except the stimulation of cyclic AMP by Δ^9-THC becomes less significant at doses of 0·25 to 1·0 mg/kg in these regions when compared to that of whole brain. Nevertheless, there is a persistently significant elevation of cyclic AMP in these regions at 0·1 mg/kg of Δ^9-THC. The values for hypothalamus did not exhibit a clear pattern, although the statistically most significant points (0·25 mg/kg and 2·0 mg/kg) suggest a trend in the opposite direction, namely depression of cyclic AMP followed by elevation.

DISCUSSION

The present results clearly demonstrate that administration of Δ^9-THC exerts an over-all biphasic effect on brain levels of cyclic AMP, with doses less than 2·0 mg/kg inducing an elevation of cyclic AMP levels and doses of 2·0 mg/kg and greater causing a depression of cyclic AMP. The existence of a secondary stimulation of cyclic AMP levels at 1·0 mg/kg in the whole brain indicates a complex interaction of Δ^9-THC with the cyclic AMP regulatory systems, and suggests that more than one mode of action may be involved.

These general results can be correlated with observations of other investigators on the effects of Δ^9-THC on brain levels of biogenic amines. For example, Holtzman *et al.*[17] observed that serotonin levels were increased and norepinephrine levels were decreased by intraperitoneal injections of Δ^9-THC in the dose range of 5–50 mg/kg. Using intravenous injection, Ho *et al.*[16] also found that relatively high doses of THC cause an elevation of serotonin and depression of norepinephrine levels. However, with smaller doses (less than 2 mg/kg) they observed the opposite effects, namely a decrease in serotonin and an elevation of norepinephrine. Thus, the presently observed biphasic effect of THC on cyclic AMP levels is parallel to the effects of THC on brain norepinephrine levels, and is inversely related to THC-induced changes in serotonin levels.

Biphasic effects of THC have also been observed on other parameters. For example, Sofia[43] has reported a biphasic effect of THC on rectal temperature in rats, with low doses (0·5 to 1·0 mg/kg) producing hyperthermia and high doses (4–8 mg/kg) causing a marked hypothermia. Biphasic effects of THC on behavioral reponses have also been reported, with low doses causing excitation and increased response rate, while higher doses produce decreased motility and suppression of response rate.[44–46]

The similarity of these reported biphasic effects of THC on biogenic amines, temperature regulation, and behavior with the presently reported observation of a biphasic effect of THC on brain cyclic AMP levels suggests that these findings may be all interrelated. Especially significant is the observed correlation with behavioral responses, since it is changes in behavior induced by THC which are most directly rele-

vant to any discussions of the effects of marihuana in man. These data suggest, then, that the THC-induced changes in cyclic AMP may be involved in the behavioral effects of this drug. There is some direct support for this conclusion available in the studies of Gessa *et al.*,[30] who demonstrated that microinjection of dibutyryl cyclic AMP into the lateral ventricles or the hypothalamus produces psychomotor stimulation, while injection into the recticular formation causes catatonia. It is interesting to note that these behavioral patterns are similar to those described by several investigators as occurring over time after a high initial dose of THC,[2,17,47] namely excitation followed by catatonia or depression. Other indirect evidence suggesting a relationship between psychological state and cyclic AMP can be found in the reports that urinary excretion of cyclic AMP is elevated in manic patients and lowered in patients with severe depression.[48,49]

The present data raise the obvious critical question as to the mechanism via which THC induces an alteration of cyclic AMP levels. One can distinguish at least two general levels at which THC might be acting. One possibility would be an indirect effect resulting from an alteration in biogenic amine metabolism and/or transport. Since biogenic amines are known to stimulate the formation of cyclic AMP, any changes in biogenic amines caused by THC could, in turn, affect cyclic AMP levels. In addition to this relatively indirect mode of action, the other general way in which THC might be acting would be directly on the enzymes involved in cyclic AMP formation and breakdown, namely adenyl cyclase and phosphodiesterase.

In regard to the first possibility, namely an indirect mechanism of action, evidence is now available which suggests that THC can inhibit the uptake and turnover of serotonin and norepinephrine in brain particulate fractions.[50,51] Although data is available which suggests that THC does not affect at least one enzyme of biogenic amine metabolism, namely monoamine oxidase,[18] data for other enzymes are not available. In regard to the second possibility of direct effects of THC on the cyclic AMP system itself, changes in adenyl cyclase and phosphodiesterase in response to THC have not yet been observed. Since a resolution of THC-induced alterations in cyclic AMP may be of major importance for understanding marihuana action, experiments along these lines are currently being pursued in our laboratory.

Acknowledgements—We wish to thank Dr. Monique Braude of the Center for Studies of Narcotic and Drug Abuse, National Institute of Mental Health, for generously supplying us with the cannabinoids used in these studies. The figure was prepared by Sandra Beadle. Assistance in some of the assays was provided by Ariela Goldschmidt.

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