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THE EFFECTS OF HASHISH COMPONENTS AND THEIR MODE OF ACTION ON MONOAMINE OXIDASE FROM THE BRAIN

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Abstract—Cannabinoids apparently interfere with the metabolism of biogenic amines in the brain, indicating that the biogenic amine system is involved in the psychomimetic effect of hashish. The effect of Δ^1-tetrahydrocannabinol (Δ^1-THC), cannabidiol (CBD) and hashish extract on monoamine oxidase (MAO) from porcine brain mitochondria was therefore studied, using several substrates including amines of biological importance, such as serotonin, dopamine and tryptamine. While Δ^1-THC and hashish extract inhibited MAO activity, to an extent varying according to the substrate, CBD was innocuous or only slightly inhibitory—in correspondence with the psychomimetic potency. Phospholipids associated with MAO were essential for the inhibition of the oxidase activity by Δ^1-THC. In enzyme preparations from which the phospholipids were extracted, the sensitivity to Δ^1-THC essentially disappeared. Sensitivity could be regained specifically upon addition of phosphatidylcholine. It is suggested that the phospholipids of the MAO complex function as the site of interaction with the cannabinoids, leading to the observed changes in MAO activity. The differential effect of the cannabinoids support the conclusion that MAO plays a central role in mediating the psychomimetic effect of hashish.

Biogenic amines have been implicated in the expression of the psychoactivity of hashish and marihuana [1–4]. Increased levels of biogenic amines and catecholamines were found in experimental animals *in vivo* after administration of hashish extract or Δ^1-tetrahydrocannabinol (Δ^1-THC), the psychoactive component of hashish. Several mechanisms may account for these elevated levels: (a) Changes in the uptake rates of these compounds into their sites of action, (b) changes in their turnover rate, (c) changes in their biosynthesis or metabolism rate.

Monoamine oxidase (MAO), an enzyme embedded in the outer membrane of the mitochondrion, has an important role in the metabolism of endogenous monoamines in the brain [5] and as such may be considered as one of the possible sites for the action of hashish. Recently, it was shown in our laboratory that Δ^1-THC and hashish extract inhibit MAO activity of brain mitochondria *in vitro* with benzylamine or tyramine as the substrates, while cannabidiol (CBD), a non psychoactive component of hashish, did not have this effect [6]. Such a differential response is intriguing in view of the multiplistic effects of cannabinoids on biological membranes, with little or no selectivity in the drug potency. Membranal functions of human and rat red blood cells [7, 9] rat liver mitochondria [10, 11], rat liver lysosomes [12] and artificial lipid vesicles [13] are affected by various cannabinoids, but none of these systems can clearly differentiate between the active and the nonactive compounds of cannabis. In examining further the effects of cannabinoids on MAO activity, we included in the present study substrates of biological and behavioral importance, such as 5-hydroxytryptamine (5-HT, serotonin), 3-hydroxytyramine (dopamine) and tryptamine.

MAO is a lipoprotein which contains some 20 or more moles of phospholipids per mole of protein [5]. In the present study we also examine an hypothesis that these phospholipids may function as the site of interaction of the lipophylic Δ^1-THC with the MAO complex, possibly accounting for the inhibition of the enzyme activity.

MATERIALS AND METHODS

Preparation of mitochondria. Porcine brain mitochondria were prepared according to Tipton [14], with the exception that the mitochondria (protein concentration of 10 mg/ml) were stored frozen in sucrose 0.25 M pH 7.6. This preparation is a crude mitochondrial MAO.

Solubilization of monoamine oxidase. The mitochondrial preparation (stored frozen overnight) was allowed to thaw at room temperature, and the suspension was then sonicated in a MSE sonifer 100 W at an amplitude of 8 microns peak to peak, for 15 min. The sonicate was then centrifuged at 100,000 *g* for 120 min. Temperature was kept under 4° through all the treatments. The supernatant (soluble MAO) was decanted and used for testing enzyme activity.

Assay of MAO activity. Three ml of the reaction mixture contained: potassium phosphate buffer pH 7.4, 50 mM, the enzyme preparation at 0.5–1.0 mg of protein, and 1 mM substrate. The reaction was initiated with the substrate, usually benzylamine, and took place at 37°. Five min after commencement of the reaction, 1.5 ml from each test tube were transferred to another test tube that contained 0.25 ml, 10% $ZnSO_4$, and 0.05 ml 1N NaOH were added. This test tube was used as zero time reading. The remaining 1.5 ml of reaction mixture were incubated

at 37° and the reaction was terminated 60 min later by the additions of $ZnSO_4$ and NaOH solutions. All test tubes were then dipped in boiling water for 2 min, followed by 10-min centrifugation at 1000 *g*. When serotonin, dopamine or tryptamine were the substrates 0.15 ml, 20% trichloroacetic acid were added to 1.5 ml reaction mixture samples to terminate the reaction, followed by centrifugation. Each supernatant was read against its zero-time supernatant, using a double beam spectrophotometer to reveal the presence of the corresponding aldehydes. The rate of product formation proceeded linearly for the duration of the assays (1 hr).

Determination of aldehydes. Benzylamine oxidation was followed by the method of Tabor *et al.* [15]. Dimethylaminobenzaldehyde was determined by the method of Dietrich and Erwin [16]. When tyramine was used as substrate, the Method of Schurr and Livne [6] was used. When 5-HT, serotonin, dopamine or tryptamine were used, the corresponding aldehydes were determined by the following method: to 1 ml of the supernatant 0.1 ml dinitrophenylhydrazine, 25 mM (in methanol), and one drop of concentrated HCl were added. The mixture was incubated at 50° for 30 min. Then 2.5 ml of NaOH, 2N, were added after cooling. Spectrophotometric readings were made immediately at wavelengths of 530,425 and 500 nm for serotonin, dopamine and tryptamine oxidation products, respectively. By simultaneous spectrophotometric assay and an assay based on the formation [^{14}C]5-hydroxyindolacetaldehyde from [^{14}C]serotonin [5], the ϵ_m of the product was determined to be 2.4×10^4. No such assays were made for dopamine and tryptamine and the results presented with these substrates are in arbitrary units.

Effect of cannabinoids. Aliquots of ethanolic stock solutions of hashish extract, Δ^1-THC or CBD were mixed with 3 ml of the assay buffer and the MAO preparation was then added as described above. Ethanol was included in the control tubes at the same final concentration (0.33% v/v). Following a preincubation period of 30 min at 37°, the reaction was initiated by adding the substrate.

Chloroformic extraction of soluble MAO preparation. To a given volume of soluble MAO preparation, Δ^1-THC or CBD were added to a final concentration of about 30–60 μg per 1 mg of protein. In the controls ethanol replaced Δ^1-THC or CBD. After an incubation of 30 min at room temperature, the mixture was extracted with 3 vol. of cold chloroform by vigorous mixing for 5 min. Following centrifugation at 10,000 *g* for 20 min the upper aqueous layer was collected, a stream of N_2 was passed over it to assure the removal of chloroform, and then the preparation was assayed for enzyme activity. This extraction must take place no later than 24 hr after the sonication step.

Preparation of liposomes. Liposomes were prepared according to Alhanaty and Livne [13] in potassium phosphate buffer, 50 mM, pH 7.4 using various phospholipids.

Hashish extract. Hashish was extracted with petroleum ether as described elsewhere [6].

Compounds used. Δ^1-THC and CBD were purchased from Makor Chemical Co., Jerusalem. All substrates and phenylcyclopropylamine (SKF 385) were obtained from Sigma Chemical Co., St. Louis. [^{14}C]Serotonin creatinine-sulphate was from Amersham. Phosphatidylcholine was extracted and purified from egg yolks according to Dawson [17]. Cardiolipin was obtained from Sigma Chemical Co. and phosphatidylserine and phosphatidic acid from Supeleo Inc., PA. Dinitrophenylhydrazine was obtained from B.D.H. Chemical Co. Tritiated Δ^1-THC and CBD were kindly supplied by NIDA, Bethesda, MD. All other compounds used were of analytical grade.

Data presented. Each of the experiments was repeated at least four times in duplicates, yielding identical patterns. Duplicates agreed within 5 per cent experimental error, and representative experiments are shown.

RESULTS AND DISCUSSION

MAO activity of brain mitochondria in systems containing various substrates was inhibited by Δ^1-THC and hashish extract while CBD was found to be innocuous or only slightly inhibitory (Fig. 1). Different degrees of inhibition with Δ^1-THC were found for the different substrates, ranging from about 80 per cent inhibition when dopamine was the substrate to about 25 per cent when tryptamine was used. Hashish extract was inhibitory at a much lower dose and its effect varied as well according to the substrate tested. Phenylcyclopropylamine (SKF-385), a known competitive inhibitor of MAO, was used for a comparison, at a final concentration of 3.3×10^{-6} M.

Study of the kinetic parameters of the enzyme activity in the presence of different concentrations of Δ^1-THC and substrates, revealed that the inhibition produced was of the noncompetitive type, independent of the substrate used (Fig. 2). This phenomenon suggests an indirect effect of the inhibitor on the enzyme's active site, probably through a lipophylic component in the mitochondrion outer membrane or through a moiety of the enzyme itself.

If the interaction of Δ^1-THC with MAO takes place through a membranal component, separation of the enzyme from the membrane by means of solubilization should abolish the inhibitory effect of the cannabinoid. If, on the other hand, Δ^1-THC affects MAO activity directly or through a phospholipid moiety of the enzyme itself, then the cannabinoid must also affect the soluble enzyme, although no membrane or membranal particles are present. To solubilize the enzyme, the technique of Tipton [14] was adopted, mainly because it avoids the use of detergents, which are very difficult to remove after the solubilization. The soluble enzyme thus obtained was found to be inhibited by Δ^1-THC, as Table 1 (Treatment I) shows.

On the basis of this finding one could assume that the component which is responsible for the interaction of Δ^1-THC with MAO is part of, or bound to, the enzyme itself. MAO phospholipids [5] are likely to serve this function and therefore attempts were made to extract the phospholipids from the enzyme. Table 1 summarizes the various

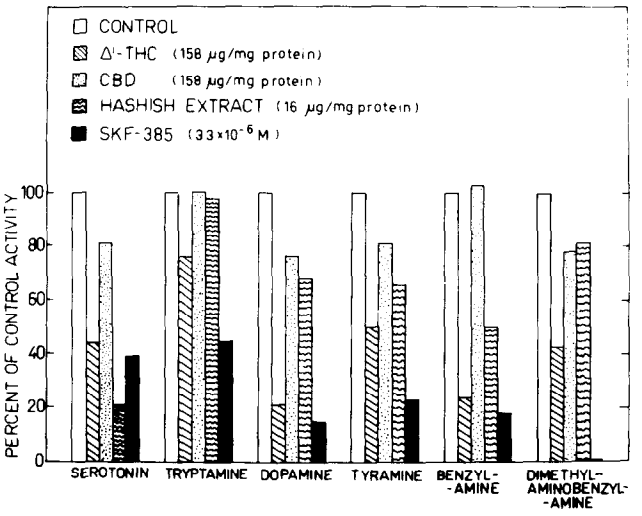

Fig. 1. The effects of Δ¹-THC, CBD, hashish extract and SKF-385 on MAO activity in the presence of 1 mM of various substrates (tryptamine was present at 5 mM). 100% of control activity is the enzyme activity in the presence of each substrate and in the absence of any inhibitor.

treatments carried out for this purpose. As can be seen, the extraction alone, irrespective of the solvent used (Treatments II, III) did not abolish the inhibitory effect of Δ¹-THC.

It has been shown by Sklenovsky *et al.* [18] that Δ¹-THC causes an increased disruption of firm complexes of phospholipids from brain tissue and thus increases the extractability of these substances. Following Treatment IV (Table 1), when soluble MAO was preincubated with Δ¹-THC (see Methods) and then extracted with chloroform, the inhibitory effect of Δ¹-THC was indeed drastically reduced. Since CBD is capable of competing with Δ¹-THC, probably on the very same site on the MAO molecule [6], it might cause the "labilization" of the phospholipid complexes similarly to Δ¹-THC. Treatment V in Table 1 clearly indicates that this is the case. To probe whether the cannabinoids incubated with the enzyme were extracted to the chloroform phase, we used tritiated Δ¹-THC or CBD in this procedure. The results show that up to 3 per cent of the cannabinoids remained within the aqueous phase after the chloroformic extraction.

The resolution thus obtained between the MAO and the extractable factor, which is responsible for the enzyme sensitivity to Δ¹-THC, enabled us to

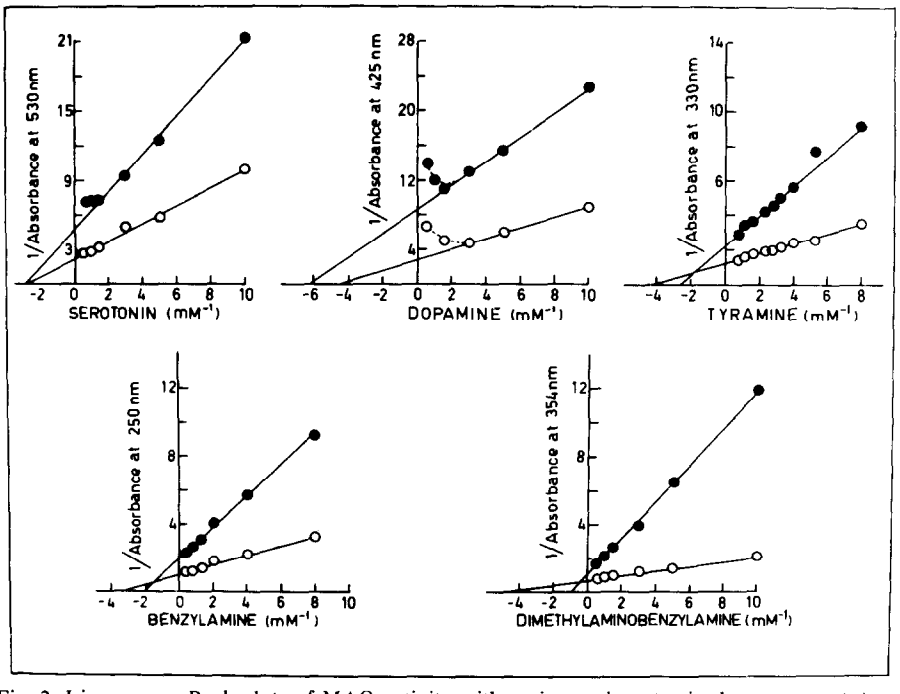

Fig. 2. Lineweaver–Burk plots of MAO activity with various substrates in the presence (●) or absence (○) of 158 μg Δ¹-THC per mg of protein.

Table 1. The effects of Δ^1-THC and CBD on MAO activity in soluble preparation before and after various organic extraction procedures

Treatment	MAO activity* Control	Δ^1-THC†	CBD†
I None	1.00	35	88
II 2-Butanone extraction‡	0.76	32	82
III Chloroformic extraction	0.75	36	86
IV Chloroformic extraction in the presence of Δ^1-THC	0.75	81	169
V Chloroformic extraction in the presence of CBD	0.73	100	155

* MAO Activity in the control: benzylamine oxidized (nmoles/min/mg protein). In the presence of the cannabinoids: per cent (%) of the control.

† The cannabinoids dosage: 50 μg per mg protein.

‡ The extraction was done according to Olivecrona *et al.* [19].

examine the capacity of various phospholipids to restore the sensitivity of MAO to Δ^1-THC by adding these phospholipids, one by one or in combination, to the extracted enzyme (Fig. 3). It has already been shown that solubilized MAO from pig liver could be reassociated with phospholipids such as cardiolipin, phosphatidylethanolamine and phosphatidylinositol, but not with phosphatidylcholine [19, 20]. As can be seen from Fig. 3, phosphatidycholine reconstituted the inhibitory effect of Δ^1-THC on brain MAO activity, in contrast to its inability to reassociate with liver MAO [19, 20]. Moreover, phosphatidylcholine increased the MAO activity to the level of the untreated (soluble) enzyme and even higher. These effects of phosphatidylcholine were found to be concentration-dependent as can be seen from Fig. 4. The optimal concentration was 1.1 mg/mg of protein, while lower and higher concentrations were less effective. Other phospholipids examined were much less effective, as can be seen from Fig. 3.

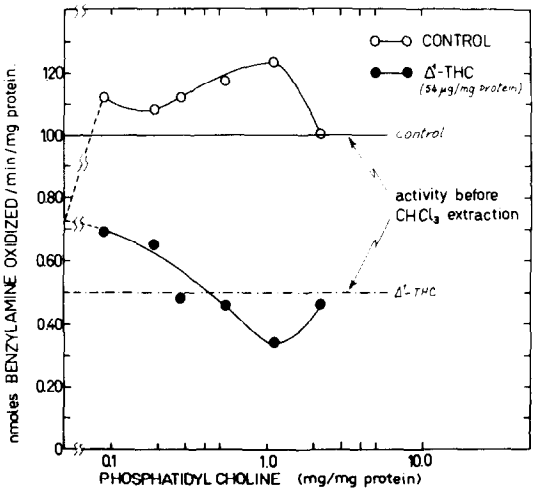

Fig. 4. The effect of added quantities of phosphatidylcholine on the activity of soluble MAO, extracted according to Treatment V of Table 1, and its sensitivity to Δ^1-THC.

On the basis of the results presented, a schematic model was drawn which outlines our proposal for the mode of action of Δ^1-THC in inhibiting MAO activity of brain mitochondria (Fig. 5). The dotted square is the polypeptide chain, the open circles are phospholipids and the triangle "S" represents the substrate. When CBD or Δ^1-THC are added, they interact with the phospholipids. While CBD causes no critical change, conformational or other, in the enzyme by this interaction (B_1), possibly because of its more flexible structure, Δ^1-THC, with its more rigid structure, causes a change which in turn decreases the enzyme activity (B_2). When soluble MAO (A) is treated with chloroform, to give form D, the phospholipids are not extractable unless the enzyme is preincubated with CBD or Δ^1-THC. The extracted enzyme (C) is not inhibited by Δ^1-THC. Phospholipids, particularly phosphatidylcholine, could reassociate with the extracted enzyme to

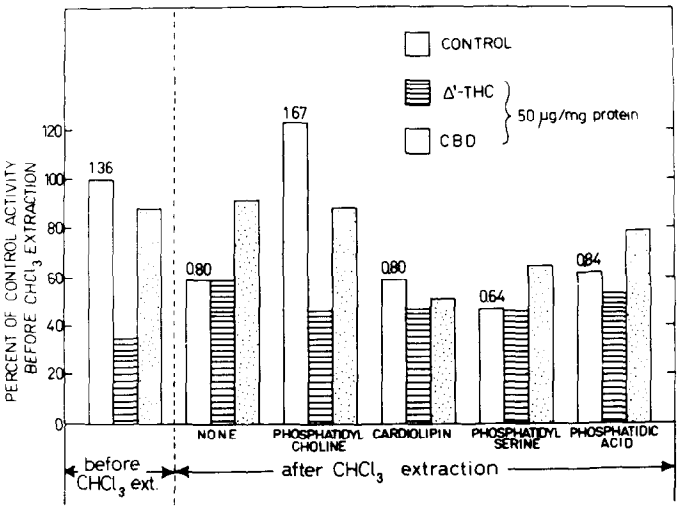

Fig. 3. The sensitivity of soluble MAO to Δ^1-THC and CBD before and after chloroformic extraction (Treatment V in Table 1) and in the presence of 1 mg of various phospholipids per mg of protein added after the extraction. The numbers at the heads of the columns are sp. act. (nmoles benzylamine oxidized/min/mg protein).

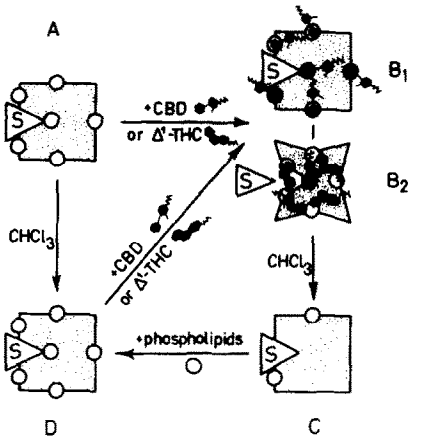

Fig. 5. Mode of action of Δ^1-THC in inhibiting MAO activity of brain mitochondria (for details see text).

reconstitute the MAO complex, which is sensitive to Δ^1-THC.

Concerning the physiological relevance of the observations presented in this study, several aspects may be emphasized. The pronounced effect exerted by hashish extract on MAO activity with serotonin as a substrate is of particular interest, in view of the significant role of serotonin in the central nerve system and the fact that the smoked material is hashish rather than the pure Δ^1-THC. We have already demonstrated the tissue selectivity with regard to MAO inhibition, when the brain mitochondrial enzyme is inhibited by Δ^1-THC and hashish extract, while that of the liver is not [6]. Furthermore, it is possible to mimic known *in vivo* effect of cannabinoids such as the antagonistic action of CBD to that of Δ^1-THC [21, 22] with the MAO system prepared from brain mitochondria [6]. It is thus concluded that MAO of brain mitochondria plays an important role in the psychoactivity of hashish.

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