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On the Action of Δ^9-Tetrahydrocannabinol as an Inhibitor of Sodium- and Potassium-Dependent Adenosine Triphosphatase

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SUMMARY

TORO-GOYCO, EFRAÍN, RODRÍGUEZ, M. B. & PRESTON, A. M. (1978) On the action of Δ^9-tetrahydrocannabinol as an inhibitor of sodium and potassium-dependent adenosine triphosphatase. *Mol. Pharmacol.*, 14, 130-137.

Chromatographically pure Δ^9-tetrahydrocannabinol (Δ^9-THC) was tested *in vitro* for inhibitory activity on sodium- and potassium-stimulated ATPase (ATP phosphohydrolase, EC 3.6.1.3) from various sources. Concentrations of 3 μM Δ^9-THC inhibited by 50% enzyme preparations with specific activities of 1200-1500 nmoles of P_i per minute per milligram of protein. Rat brain ATPases were much more sensitive to Δ^9-THC than to equal concentrations of ouabain. The kinetics of inhibition of electric eel ATPases seemed to be noncompetitive with respect to ATP. Rat brain ouabain-insensitive ATPases were also inhibited by Δ^9-THC, and preliminary kinetic data indicated that inhibition is competitive with respect to ATP. In Ehrlich ascites tumor cells (10^7 cells/flask), 60 μM Δ^9-THC inhibited nucleoside ([^{3}H]thymidine) incorporation without exerting any significant effect on the viability of cells as measured by O_2 consumption. Particulate ATPases isolated from cells exposed to Δ^9-THC were found to have significantly lower specific activity than cells in control media, 123 ± 36 compared with 172 ± 16 nmoles of P_i per minute per milligram of protein ($p < 0.005$, $n = 16$). The hypothesis is advanced that several of the observed physiological effects of Δ^9-THC can be explained on the basis of its ATPase-inhibitory activity.

INTRODUCTION

Any study leading toward the elucidation of the mechanism of action of Δ^9-tetrahydrocannabinol is of interest for a variety of reasons. The chemical is the object of intensive studies because of its diversity of pharmacological effects. Δ^9-THC[1] has potential therapeutic value as an analgesic (1). It has also been shown to possess antitumor properties (2), to inhibit the uptake of nucleosides and amino acids by lymphocytes (3), to inhibit protein and nucleic acid synthesis (4, 5), and to antagonize the effects of insulin (6). In spite of these findings, no clear indication as to its possible mechanism of action is yet available.

Inhibition of cell and thus tumor growth can be achieved by diminishing the availability of nutrients to rapidly metabolizing cells. Optimal concentrations of the necessary nutrients become available to cells by various transport processes. If the unifying concept that the active pumping

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[1] The abbreviation used is: Δ^9-THC, Δ^9-tetrahydrocannabinol (also known as Δ^1-tetrahydrocannabinol).

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of a single substance out of the cell (e.g., sodium) may furnish the driving force for the active transport of a variety of other substances into the cell is valid, then the inhibition of the enzymes involved in sodium transport may have far-reaching effects on the transport of other nutrients and on all other biosynthetic processes in the cells. One of these membrane-bound enzymes is the sodium- and potassium-stimulated ATPase (ATP phosphohydrolase, EC 3.6.1.3). With these considerations in mind, we decided to study the action of Δ^9-THC on this enzyme isolated from various sources.

MATERIALS AND METHODS

ATP (disodium salt) and ouabain octahydrate were obtained from Sigma. Stock solutions of ATP were prepared by weighing the desired amount of the chemical, dissolving it in about half the final volume of water, neutralization to pH 7.2 with 1 M Trizma base with a pH-stat (Radiometer), and adjustment to the desired volume. These solutions were kept frozen in small aliquots until used. Ouabain was stored under refrigeration as a 10 mM solution in water until used. [*methyl*-^{3}H]Thymidine with a specific activity of 40–80 Ci/mmole was purchased from ICN Isotope and Nuclear Division. Minimum essential medium without glutamine, with Hanks' salts, was purchased from Grand Island Biological Company.

Protein was determined by the method of Lowry et al. (7). Statistical analysis of the kinetic data was done by the nonlinear regression method of Wilkinson (8), using a program adapted to a model 9810. A Hewlett-Packard computer. Student's *t*-test (9) was used for statistical evaluation of the results in the experiments *in situ*, in which changes in the specific activity of (Na^+ + K^+)-ATPases of Ehrlich ascites cells exposed to Δ^9-THC were measured.

Preparation of (Na^+ + K^+)-ATPase from electric eel. One source of (Na^+ + K^+)-ATPase used for these studies was the electric organ of the *Electrophorus electricus*. The procedure was performed essentially as described by Albers *et al.* (10), with modification that the tissue was disrupted by homogenization. Forty grams of tissue were chopped into small pieces, suspended in 200 ml of cold 5 mM EDTA, pH 7.0, and homogenized at medium speed in a VirTis 23 homogenizer. The homogenate was centrifuged at 1000 × *g* for 15 min. The pellet was resuspended as above, homogenized again for 3 min at maximum speed, and centrifuged as above. The enzyme was found essentially in the supernatant. The enzyme preparations had a specific activity of 1500 nmoles of P_i per minute per milligram of protein, were 95–98% sensitive to ouabain, and were stable when lyophilized.

Preparation of ATPase from adult rat brain. Adult Sprague-Dawley rats weighing 250–300 g were killed by decapitation and their brains were removed. The cerebellum was discarded, and the enzyme was prepared using the procedure described by Ahmed and Judah (11), with the modification that sucrose was used instead of mannitol. The specific activity of these preparations was 1000–1250 nmoles of P_i per minute per milligram of protein and was 75–80% sensitive to 1 mM ouabain.

Assays for (Na^+ + K^+)-ATPases (cell-free treatment). Enzyme activity was measured as inorganic phosphorus liberated. The procedure has been described elsewhere (12). The total volume of the incubation mixture was 1 ml. In those assays where ouabain or Δ^9-THC was used as inhibitor the blanks contained the corresponding amounts of inhibitor in its respective solvent.

Δ^9-THC used in this work was chromatographically pure. One-dimensional thin-layer chromatography performed in two different solvent systems [hexane–diethyl ether–acetic acid (90:15:1) and chloroform–methanol–water (65:25:4)], followed by development in iodine vapors, showed the preparation to be free of impurities. Δ^9-THC was dissolved in a 98:2 (v/v) water–acetone mixture immediately before use. Working solutions contained 10 μg/ml.

Assays for (Na^+ + K^+)-ATPases (treatment in situ). Ehrlich ascites tumor cells grown in mice were used as a third source of (Na^+ + K^+)-ATPase. Cells were ob-

tained by killing the tumor-bearing animals and filtering the fluid containing the cells through two layers of cheesecloth. The filtrate was placed in centrifuge tubes and washed repeatedly with Hanks' salt solution until all red blood cells were removed. Once clean, the cells were counted in a counting chamber. Cell dilutions were made with Hanks' solution so that 0.1 ml contained 10^7 cells. Then 2.8 ml of minimum essential medium plus glutamine (2 mM) were placed in 10-ml flasks, 0.1 ml of a Δ^9-THC solution containing 1 mg/ml was added, and finally 0.1 ml of a cell suspension was added, making a total volume of 3 ml. Control flasks were prepared with the same contents except for the Δ^9-THC. Flasks were incubated at 37° for 50 min, after which the cells were centrifuged at 500 × *g* in a refrigerated centrifuge for 10 min. The supernatant was decanted, and both the supernatant and the cell pellet were frozen.

The pellets were resuspended in 2 ml of water and disrupted using 10 strokes in a motorized all-glass homogenizer. The homogenized cells were checked for complete disruption under a light microscope. After disruption the cells were centrifuged again at 500 × *g* for 10 min, and the pellet was tested for ATPase activity.

Oxygen consumption. To test whether Δ^9-THC had any effect on cellular respiration (O_2 consumption), cell suspensions containing 10^7 cells, prepared as described above, were incubated in Warburg flasks using a Warburg apparatus (Precision Scientific). After 10 min of equilibration and an additional 10 min of incubation to test oxygen consumption, the Δ^9-THC (50–200 μg) was added from the side arm. The solvent used for Δ^9-THC was added to the control flasks. Incubation was continued for 50 min.

[^{3}H]Thymidine incorporation as acid-insoluble radioactivity into Ehrlich ascites tumor cells (in situ). Ehrlich ascites tumor cells (10^7 cells/flask) prepared as described above were placed in sterile flasks containing the incubation mixture and were incubated for 40 min at 37°. The incubation mixture contained 0.1 ml of a solution containing various concentrations of Δ^9-THC, 0.1 ml of a thymidine solution containing 100 μCi/ml, glutamine (2 mM, final concentration), and minimal essential medium. The final volume was adjusted to 5.0 ml with minimal essential medium. The control flasks contained all the ingredients mentioned above except the inhibitor. At the end of the incubation period the mixture was poured into centrifuge tubes and 4 ml of 10% trichloroacetic acid solution were added. The tubes were centrifuged at 500 × *g* for 10 min, the supernatant was discarded, and the precipitate was solubilized with 0.5 ml of "NCS" tissue solubilizer (Amersham/Searle) and placed in counting vials with 10 ml of Tritisol prepared according to Fricke (13). Vials were counted in a Picker liquid scintillation counter.

RESULTS

Studies in vitro: effects on electric eel ATPases. Optimal conditions for the assay of (Na^+ + K^+)-ATPase activity required 150 mM Na^+, 10 mM K^+, and 5 mM Mg^{++}. Enzymatic activity decreased when the K^+ concentration was altered from its optimum value, leaving all other experimental conditions constant (Fig. 1). The inhibitory effect of Δ^9-THC was more pronounced at the optimum ionic concentrations, but did not seem to potentiate the inhibitory activity of K^+ when the concentrations of this ion deviated markedly from the optimum (0 or 40 mM). Acetone, used as solvent for the drug, in final concentrations of 0.2% in the incubation mixture, had no significant effect on the enzymatic activity.

Figure 2 illustrates the results obtained when the Na^+ concentration was varied while K^+ and Mg^{++} remained constant. Again, maximal inhibitory activity due to Δ^9-THC alone was obtained when ionic concentrations were optimal for activity.

Enzyme activity was determined in the absence and presence of Δ^9-THC at various concentrations of ATP. The double-reciprocal (Lineweaver-Burk) plot in Fig. 3 makes it evident that Δ^9-THC behaves as a noncompetitive inhibitor of the substrate. The data presented in Fig. 3 were also evaluated by the method of Wilkinson

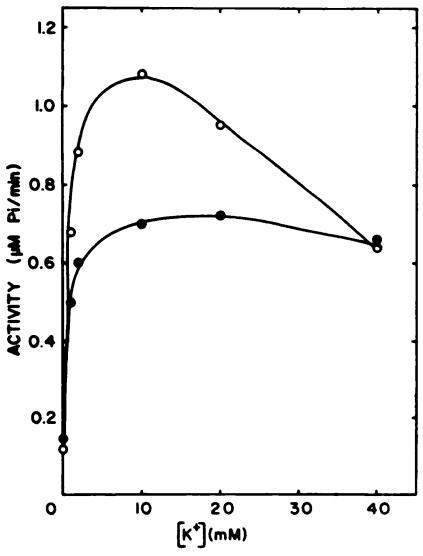

FIG. 1. *Electric eel (Na⁺ + K⁺)-ATPase activity in the absence* (○——○) *and presence* (●——●) *of 1 μg of Δ⁹-THC at various concentrations of K⁺*

Incubation was performed with optimal concentrations of Na^+ and Mg^{++} (as chloride salts). Maximum inhibition due to Δ⁹-THC occurred at 10 mM K^+. Total protein in the incubation mixture was 10 μg.

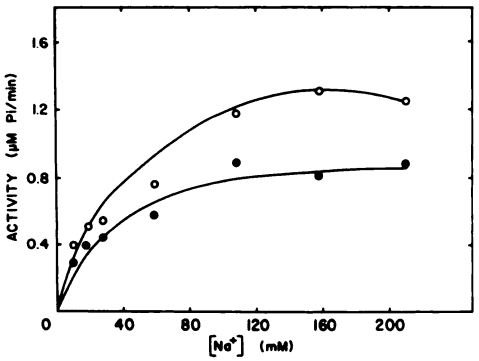

FIG. 2. *Electric eel (Na⁺ + K⁺)-ATPase activity in the absence* (○——○) *and presence* (●——●) *of 1 μg of Δ⁹-THC at various concentrations of Na⁺ and optimal concentrations of K⁺ and Mg⁺⁺*

The sharpest reduction in enzymatic activity due to Δ⁹-THC occurred at 150 mM Na^+, which was the optimal concentration for activity in the absence of the drug. Total protein in the incubation mixture was 10 μg.

(8). The calculated values for K_m in the absence (4.94 ± 1.37 mM) and presence (5.59 ± 1.87 mM) of inhibitor were not significantly different, confirming that the inhibition exerted by Δ⁹-THC on the enzyme is noncompetitive. Maximum velocity in the absence of inhibitor was 1.31 ± 0.10 μmoles/min, while in the presence of 30 μM Δ⁹-THC it was 0.98 ± 0.10, a highly significant difference ($p < 0.001$). Under the experimental conditions used in this work, addition of 2.3 μg of Δ⁹-THC to the incubation medium reduced enzyme activity by 50% (I_{50} = 70 μM) (data not shown).

Effect on brain ATPases. Table 1 shows the relative inhibitory potencies of Δ⁹-THC and ouabain in crude ATPase preparations from brain tissue. Concentrations of Δ⁹-THC as low as 1/30 that of ouabain produced inhibition of the same order of magnitude. For enzyme preparation X, the respective inhibition by ouabain and Δ⁹-THC was 61.4% and 50%. For enzyme preparation Y, the respective inhibition was 74.5% and 66.8%. Significantly, a combination of Δ⁹-THC and ouabain (3 nmoles of the former and 1000 nmoles of the latter) caused greater inhibition of ATPase activity than either inhibitor alone.

Figure 4 shows the diminution in brain ATPase activity as a function of Δ⁹-THC concentration. Inhibition of both ouabain-sensitive and insensitive ATPase activity leveled off at drug concentrations of 1 μg/ml. Kinetic data showing the differences in inhibition caused by ouabain and ouabain plus Δ⁹-THC (3 μM) are illustrated in

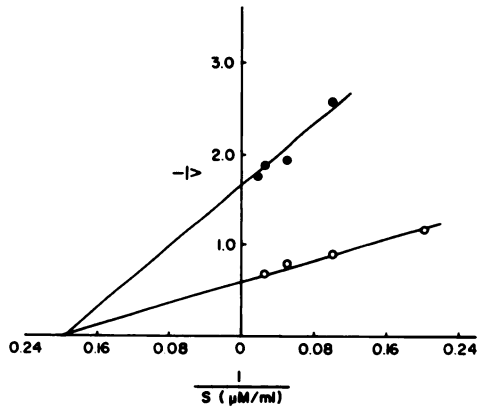

FIG. 3. *Double-reciprocal plot indicating noncompetitive effects of Δ⁹-THC on ATP*

Electric eel ATPase, 95% sensitive to ouabain, was used for this experiment. Incubation was performed using optimal concentrations of cations. For further details, refer to the text.

TABLE 1

Inhibitory activity of various substances on (Na^+ + K^+)-ATPase activity of rat brain

Assays for activity were performed as described in the text. Two different enzyme preparations (X and Y) were prepared on different dates.

Contents of mixture	Specific activity	Inhibition
	nmoles P_i/min/mg protein	%
A. Enzyme preparation X (10 μg of protein)	880	
B. A + 0.1 μmole of ouabain	340	61.4
C. A + 0.003 μmole of Δ^9-THC	442	50.0
D. A + B + C	193	78.4
E. Enzyme preparation Y (20 μg of protein)	1010	
F. E + 1 μmole of ouabain	260	74.5
G. E + 10 μmoles of serotonin	950	5.9
H. E + 0.03 μmole of Δ^9-TC	335	66.8
I. E + F + 0.003 μmole of Δ^9-THC	60	94.3

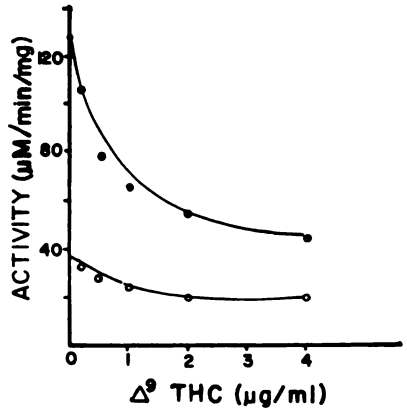

FIG. 4. *Effect of varying concentrations of Δ^9-THC on activity of brain ATPases*

●——●, total ATPase activity; ○——○, ouabain-insensitive ATPase activity. Ouabain-insensitive activity was determined as the residual activity after the addition of 1 mM ouabain to the incubation mixture.

Fig. 5. The concentration of ouabain used (1 mM) was enough to cause complete inhibition of the (Na^+ + K^+)-ATPases. The residual ATPase activity, insensitive to ouabain, was further inhibited competitively by Δ^9-THC, showing that Δ^9-THC inhibits other ATPases as well as (Na^+ + K^+)-ATPases.

Effects on (Na^+ + K^+)-ATPases from Ehrlich ascites tumor cells. Table 2 summarizes the results obtained in a series of nine experiments with their respective controls. The specific activity of the crude ATPase preparations isolated from control cells was higher than that of Δ^9-THC-treated cells (172 ± 16 compared with 123 ± 36 nmoles/min/mg of protein). The calculated t value of 3.71 makes these differences highly significant (n = 16, p < 0.005).

Effects of Δ^9-THC on incorporation of [^{3}H]thymidine as acid-insoluble radioactivity into Ehrlich ascites tumor cells. Figure 6 shows the effect of Δ^9-THC on the incorporation of [^{3}H]thymidine as acid-insoluble radioactivity into Ehrlich ascites cells. Concentrations of 100 μg/flask inhibited incorporation by 65%. As stated above, the same concentrations of Δ^9-THC were found to diminish significantly the specific activity of crude ATPase preparations isolated from the same cells.

Effects of Δ^9-THC on viability of Ehrlich ascites cells. To rule out the possibility that Δ^9-THC might impair the viability of the tumor cells, oxygen consumption by the cells was measured at 10-min intervals during the incubation period, using a War-

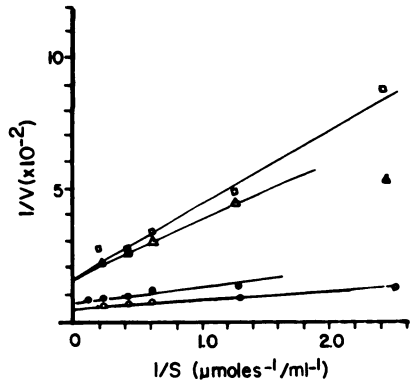

FIG. 5. *Double-reciprocal plot illustrating inhibitory effects of 3 μM Δ^9-THC on brain ATPase preparations*

○——○, (Na^+ + K^+)-ATPase activity; ●——●, (Na^+ + K^+)-ATPase activity in the presence of Δ^9-THC; △——△, ouabain-insensitive ATPase activity; □——□, ouabain-insensitive ATPase activity in the presence of Δ^9-THC. The total specific ATPase activity of the preparation was 1200 nmoles/min/mg of protein. For further details, refer to the text.

TABLE 2

Effect of 100 μg of Δ⁹-THC on activity of a crude (Na⁺ + K⁺)-ATPase preparation from Ehrlich ascites tumor cells

For each experiment 10^7 cells were used. The cells were incubated with Δ⁹-THC in 3 ml of minimal essential medium at 37° prior to their disruption. After breakage of the cells, the crude enzyme preparations were assayed for activity as described in the text.

Sample	Specific activity	
	Control	Δ⁹-THC-treated
	nmoles P_i/min/mg protein	
1	150	78
2	181	80
3	161	121
4	195	171
5	198	179
6	165	142
7	160	118
8	164	126
9	175	95
Mean ±SE	172 ± 16	123 ± 36

Degrees of freedom = 16; $t = 3.71$; $p < 0.005$.

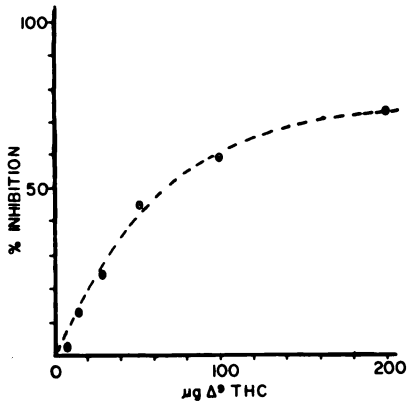

FIG. 6. *Inhibition of incorporation of [³H]-thymidine as acid-insoluble radioactivity into Ehrlich ascites tumor cells*

Δ⁹-THC concentrations causing 65% inhibition of [³H]thymidine incorporation also caused a significant decrease in (Na⁺ + K⁺)-ATPase activity in the same cells. For further details, refer to Table 2 and the text.

burg respirometer. No differences were found in the oxygen consumption of the cells exposed to Δ⁹-THC and control cells, ruling out the possibility of a significant alteration in metabolism. The explanation for the differences in incorporation of [³H]thymidine between the two groups of cells must therefore be attributable to some other mechanism.

DISCUSSION

Previous reports have shown contradictory evidence on the effects of Δ⁹-THC on ATPases. Chari-Britton and Bino (14) reported an increase in the activity of rat liver mitochondrial ATPases on exposure to Δ⁹-THC. However, results similar to the ones presented here have been obtained by Laurent *et al.* (15) for ATPases from rat ileum. This discrepancy may be partially explained by the fact that mitochondrial ATPases are not dependent on sodium and potassium.

The results presented here suggest that Δ⁹-THC is inhibitory to (Na⁺ + K⁺)-ATPases irrespective of their source, and show that ouabain-insensitive ATPases from rat brain are also inhibited by Δ⁹-THC. Electric eel ATPases used in this work (95–98% ouabain-sensitive) were inhibited noncompetitively with respect to ATP (see Fig. 3). The inhibition of ouabain-insensitive ATPases (Fig. 5) seems to be competitive with respect to ATP. The interpretation of these data, however, may be open to criticism, as ouabain-insensitive ATPases constitute only about one-fourth the total ATPase activity of these preparations.

The differences in structure between ATP as the substrate and Δ⁹-THC as inhibitor suggest that if any inhibition is to be found, it should be noncompetitive in nature. The observation of competitive inhibition by substances structurally very different from the substrate in the case of rat brain ATPases, although unusual, is not unique. Kunimoto and Umezawa (16) reported that (Na⁺ + K⁺)-ATPases isolated from rat brain are competitively inhibited by diketocoriolin·B, a sesquiterpene antitumor antibiotic isolated from fungi, and that the inhibition is reversed by phospholipids. The possibility that Δ⁹-THC, another terpenoid, might inhibit at least the ouabain-insensitive ATPases by competing with ATP merits more detailed investigation. In analogy with the work of Kunimoto and Umezawa, we have found

that phospholipids also reverse the inhibition of ATPases caused by Δ^9-THC.[2]

The inhibitory effects of Δ^9-THC on (Na^+ + K^+)-ATPases from Ehrlich ascites tumor cells (Table 2) may partially explain the observation that Δ^9-THC inhibits the incorporation of nucleosides, specifically thymidine (Fig. 6). Reduced incorporation may be due to reduced uptake.[3] The amounts of Δ^9-THC used (100 μg/flask) did not cause any significant change in the oxygen consumption of the cells, but were enough to diminish the [^{3}H]thymidine incorporation as acid-insoluble radioactivity to 35% of that seen in control media. Nahas and collaborators (3) have reported that Δ^9-THC inhibits the incorporation of nucleosides and amino acids into phytohemagglutinin-stimulated cultured lymphocytes.

The transport of many nutrients into the cell is known to be paired with Na^+ transport. A number of antitumor agents have been shown to be ATPase inhibitors, and these are known to inhibit sugar, amino acid, and nucleoside uptake by intact cells. Δ^9-THC has been reported to show antitumor properties (2) and to inhibit protein biosynthesis (4). This last process is extremely sensitive to K^+ concentration (17, 18) and ATPase activity. A reduction in the intracellular levels of K^+ or a generalized ATPase inhibition may have marked effects on the rate of protein biosynthesis.

White and collaborators (19), working with Lewis lung tumor cells grown in tissue culture, found that Δ^9-THC inhibited DNA synthesis. Their findings led them to conclude that the inhibition does not occur by depressing the precursor ([^{3}H]thymidine) uptake, because even when the drug inhibits the incorporation of the precursor into acid-insoluble material by 50%, the radioactivity in the acid-soluble pool is not affected. Those investigators did not attempt to isolate the different chemical entities containing the acid-soluble radioactivity. The incorporation of [^{3}H]thymidine as nucleotide precursor into DNA requires phosphorylation, and the energy required is supplied by ATP hydrolysis. Inhibition of ATPases in the nucleus may well inhibit the incorporation of [^{3}H]thymidine into DNA. Investigators from the same laboratory have reported that Δ^9-THC binds selectively to nuclei of mouse neuroblastoma cells (NB 2A) grown *in vitro* (20). These ATPases are known to be ouabain-insensitive. The results shown in Table 1 and Figs. 4 and 5 indicate that Δ^9-THC inhibits not only (Na^+ + K^+)-ATPases but ouabain-insensitive ATPases as well.

It is appropriate to compare the concentrations of Δ^9-THC used in this work with the concentrations to be found in habitual marijuana smokers. We used concentrations of the order of 1 μM, which are slightly above those concentrations (0.1 μM) found in the blood of subjects having smoked one marijuana cigarette (21). However, it must be taken into account that Δ^9-THC is an extremely lipophilic substance and that lipid-rich membranes and, more specifically, nervous tissue will bind the drug more avidly. Thus concentrations of 1 μM cannot be considered very far removed from those expected in habitual marijuana smokers.

It can be argued that Δ^9-THC exerts its effects by a diversity of mechanisms or, alternatively, that one mechanism of action may explain a diversity of observed effects. We have shown that ATPases are susceptible to inhibition by Δ^9-THC regardless of their source. One hypothesis advanced here is that several metabolic effects arising from alterations in intracellular ionic gradients can be explained on the basis of the ATPase-inhibitory action of Δ^9-THC. Further work to elucidate the mechanism of inhibition of (Na^+ + K^+)-ATPases by Δ^9-THC is in progress in our laboratory.

[2] E. Toro-Goyco, unpublished observations.

[3] Preliminary work in this laboratory by Drs. A. M. Preston and H. Jering using Ehrlich ascites and hepatoma cells shows that Δ^9-THC definitely decreases the uptake of [^{3}H]thymidine in both types of cells.

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