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Influence of Marihuana on Cellular Structures and Biochemical Activities

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TAHIR, S. K. AND A. M. ZIMMERMAN. *Influence of marihuana on cellular structure and biochemical activities*. PHARMACOL BIOCHEM BEHAV **40**(3) 617–623, 1991.—Cannabinoids are known to affect a number of cellular systems and functions, but the basis for their action is unclear. In this paper we review the current evidence describing cannabinoid effects on various levels of cellular structure and activity and we present our current studies on the influence of delta-9-tetrahydrocannabinol, cannabidiol and cannabinol on one cellular system, the cytoskeleton. The organization of two cytoskeletal structures, microtubules and microfilaments, were examined and the mRNA levels of tubulin and actin, the major protein components of microtubules and microfilaments, respectively, were analysed.

Tubulin Actin Cytoskeleton Cannabinoids Delta-9-tetrahydrocannabinol Gene expression Microtubules Microfilaments

CANNABINOIDS are a unique pharmacological class of compounds which are known to affect many biological systems (12, 16, 26). Because marihuana is an extensively abused substance, considerable emphasis has been placed on understanding the adverse effects of this substance; recently,the potential therapeutic uses of cannabinoids have received considerable attention (31). The mechanism(s) by which delta-9-tetrahydrocannabinol (THC) and other cannabinoids exert their effect are slowly being resolved. However, there are many outstanding questions relating to the action of cannabinoids at the cellular and molecular level. In our laboratory, the effect of cannabinoids on the cytoskeleton and on actin and tubulin gene expression, using Chinese hamster ovary cells (CHO) as a model system, is currently under investigation. By studying cannabinoid-mediated effects on various cell structures and at different levels of biochemical activity (i.e., transcription), we will be better able to explain the basis of cannabinoid action on biological systems. It should be emphasized that caution should be exercised when extrapolating data from cannabinoid action in cell cultures to the action of cannabinoids in animals or humans beings. However, an understanding of drug-induced action at the cellular level will provide a basis for evaluating drug-induced effects at the systemic or organismic levels.

Recent studies have shown cannabinoids adversely affect cytoskeletal components (7,21). The cytoskeleton is involved in a number of cellular biological processes and structures important for cell proliferation and function (13,44). The cytoskeleton forms cellular structures such as the mitotic apparatus and cilia, it interacts with cell membranes and chromosomes, and it plays an important role in many cellular activities such as cell division, macromolecular synthesis, cell motility, intracellular transport and chromosomal movement (15,46). Any alteration to the cytoarchitecture, either at the level of assembly or on the synthesis of cytoskeletal proteins, can adversely affect other biological processes (51).

In this paper we review the effects of cannabinoids on cellular systems and present results from our current research on the effects of three cannabinoids, THC, cannabinol (CBN), and cannabidiol (CBD) on cell proliferation, on the organization of two cytoskeletal elements, microtubules and microfilaments, and the change in tubulin and actin mRNA levels. For the purpose of this review, THC refers to (−)trans delta-9-tetrahydrocannabinol using the dibenzopyran nomenclature.

Cannabinoid Effects on Growth and Proliferation

Overview. Cannabinoids have been found to have an effect on cell growth, proliferation and division in a variety of cell types. Cannabinoid-induced reduction of cell growth and division has been observed in protozoans. A decrease in growth and a delay in cell division in division-synchronized *Tetrahymena pyriformis* was observed following THC (3.2–24 μM) exposure (29,53). THC and CBN depressed the growth and differentiation of the slime mold *Dictyostelium discoideum* (5), and THC and its analogues were shown to reduce growth of *Naegleria fowleri* (41).

Other cell systems sensitive to cannabinoid-induced effects on cell growth include HeLa cervical carcinoma cells (32, 39, 49), Lewis lung carcinoma cells (9) and neuroblastoma cells (7). A dose-dependent decrease in growth was reported in exponentially growing HeLa cells treated with THC, delta-8-THC, 11-OH-delta-9-THC and CBN (32). Lewis lung carcinoma tumor growth was inhibited in vivo by THC, delta-8-THC, and CBN, however, CBD appeared to have a stimulative effect on tumor growth (9). In B103 neuroblastoma cells, growth was inhibited in a dose-dependent manner by THC as evidenced by a decrease in doubling time, saturation density and a decrease in plating efficiency (7).

Marihuana smoke on sperm, T-lymphocytes, cultured lung cells and human lung and human breast cancer has been studied and extensively reviewed (23). More recently the effects of marihuana smoke and THC on murine sarcoma 180 tumor growth have been investigated (52). Cannabinoids have been shown to have an effect on the growth and differentiation of cells connected with the immune system (1, 20, 34, 35, 42, 48). For example, in vitro treatment of mitogen-stimulated cultured human lymphocytes with THC (1–100 μM range) lowered their blastogenic response (1,35). Recent studies showed that lymphocyte blastogenic transformation following stimulation with phytomitogens was inhibited by cannabinoids. THC and its metabolite 11-OH-THC inhibited human T lymphocyte blastogenesis stimulated with phytohemagglutinin and concanavalin A (48), and had differential effects on the proliferation of murine spleen, lymph node and thymus cells in vitro (42). THC has been also shown to suppress the proliferation of lymphocytes stimulated by interleukin 2 as well as the activation of lymphokine-activated killer cells (20).

Cannabinoid effects on CHO proliferation. In our laboratory, plating efficiency was used to assess the dose-dependent effects of THC, CBD and CBN on CHO viability and proliferation. The CHO cell line AuxB1 (graciously supplied by Dr. V. Ling, Ontario Cancer Institute, Toronto, Canada) was routinely grown in monolayers at 37°C in a humidified atmosphere with 5% CO_2. CHO cells were ideal for our studies because they grow rapidly, they have a short doubling time (12–18 hours), they have a high plating efficiency, and extensive literature exists describing the cytoskeletal system and the regulation of cytoskeletal protein in CHO cells (10,38). The cells were maintained in α-minimum essential medium (MEM) supplemented with 5% fetal calf serum and antibiotics (24). To assay cell proliferation, cells were resuspended from monolayers by trypsin digestion, plated in triplicate in 24-welled tissue culture plates to yield 3×10^2 cells per well, and the medium containing increasing concentrations of each drug was added to each well. Cannabinoids were suspended in an ethanol, dimethyl sulphoxide (DMSO) vehicle (21). After a 2 h exposure to the drugs or vehicle (0.012% ethanol, 0.5% DMSO, equivalent to the vehicle used with the maximum drug concentration), the medium was replaced and the cells were incubated for an additional 5–7 days to allow for colony formation. The relative plating efficiency was determined by expressing the number of colonies formed in drug-treated cells as a percentage of the number of colonies formed in untreated controls.

A representative dose response of CHO cells to THC, CBD and CBN for 2 h is shown in Fig. 1. The absolute plating efficiency (total number of colonies formed/total number of cells plated) of CHO cells varied from 60–80%. The relative plating efficiency profiles of CHO cells were similar for all 3 drugs. In general, there was little change in proliferation relative to untreated controls at cannabinoid concentrations at or below 10 μM. In cannabinoid concentrations above 10 μM, there was a decrease in the proliferative capacity of the cells. Cell proliferation decreased from 87% at 10 μM to no growth at 40 μM THC. Cell proliferation decreased from 82% and 89% at 10 μM CBD and CBN, respectively, to 9% at 40 μM CBD and CBN. Although many of the colonies that formed at concentrations above 10 μM were smaller in size and irregular in shape as compared to colonies that formed at or below 10 μM cannabinoid treatment, they were counted in our assessment. No cell proliferation was observed at 80 μM THC, CBD or CBN. The proliferation of vehicle-treated cells was similar to untreated controls.

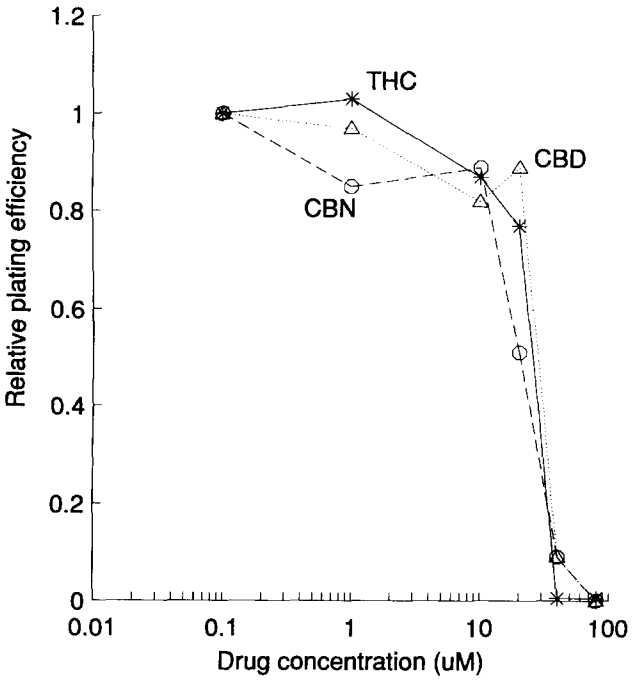

FIG. 1. The plating efficiency of CHO AuxB1 cells to THC, CBD and CBN. An equal number of cells were plated in triplicate onto 2.0 cm^2 wells and treated with cannabinoids for 2 h. Macroscopically visible colonies were counted 5–7 days after drug treatment. Plating efficiency for drug-treated cells was expressed relative to untreated controls. No difference in plating efficiency was observed between untreated and vehicle control cells.

Cannabinoid Influence on Cell Structures

Overview. It has been shown by ^{2}H-nuclear magnetic resonance and X-ray diffraction studies that cannabinoids interact with the cell membrane (25,28). Cannabinoids are highly lipophilic molecules, and this high lipophilicity leads to cannabinoids partitioning into the lipid phase of biological membranes. Cannabinoids have been shown to interact with artificial and natural membranes, and it has been suggested that some of the nonspecific effects of cannabinoids may be due to their interaction with the lipid membrane (26). Specific cannabinoid effects may be due to their interaction with other components in the membrane. It has been shown that cannabinoids affect the function of membrane bound enzymes, and the effects appear to show some stereospecificity (6,18). THC has been shown to reduce Ca^{2+}ATPase activity in gonadal tissues in mice (11). Cannabinoid inhibition of adenylate cyclase (17,45) as well as stimulation of phospholipase A_2 activity has been demonstrated (6,47). Recently, it has been reported that a receptor for THC has been identified and cloned (27).

Cannabinoids have been shown to interact with subcellular structures such as mitochondria (30), lysosomes (43) and chromosomes (50). The current evidence available suggests that cannabinoids are weak clastogens; however, the effect of cannabinoids on chromosomal breaks remain controversial (22,33). It has been suggested that errors observed in chromosome separation may reflect cannabinoid interactions on the mitotic apparatus (55). If, indeed, cannabinoids affect the mitotic mechanism, one might speculate that the structures forming the mitotic apparatus, i.e.,

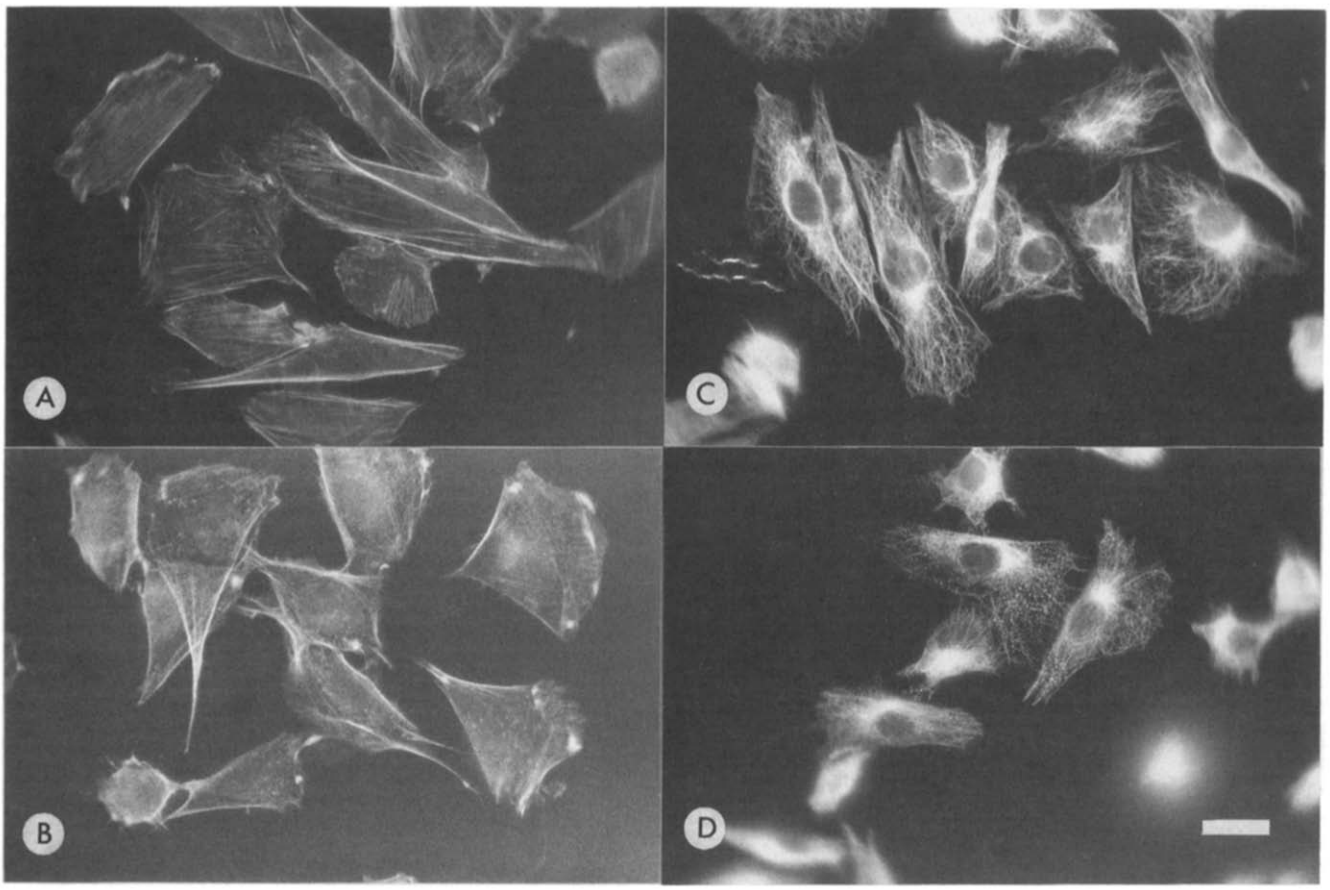

FIG. 2. Fluorescence micrographs of CHO cells stained for F-actin or tubulin. Cells were treated with 10 μM THC for 2 h. The cells were fixed/lysed simultaneously with 0.1% glutaraldehyde, 2% paraformaldehyde and 0.5% triton X-100. Cells were either stained with Bodiby-phallacidin to stain microfilaments or incubated with a primary antitubulin rat IgG followed by a FITC conjugated goat anti-rat IgG for identification of microtubules. (A) F-actin stained control cells; (B) F-actin stained cells treated with 10 μM THC; (C) Tubulin stained control cells; (D) Tubulin stained cells treated with 10 μM THC. Scale bar is 10 μm.

the microtubules, are affected in some way. Preliminary studies have shown that THC reduced birefringence of mitotic apparatus isolated from sea urchin zygotes and disrupted tubulin polymerization and depolymerization in vitro (40). More recently, a reorganization of microtubules, microfilaments and neurofilaments were reported in B103 neuroblastoma cells following THC (0.1–100 μM) treatment (7). In B103 cells, the change in the cytoskeleton corresponded with changes to the overall morphology of the cells. We have also reported that there was a disruption of stress fibers in cultured PtK_2 cells at 10 μM THC and in rabbit aortic endothelial cells following 80 μM THC treatment (21). However, no morphological changes in cell shape were observed at the lowest concentrations which disrupted stress fibers, suggesting THC may directly affect stress fibers or other mechanisms involved in microfilament assembly/disassembly.

Cannabinoid influence on microtubule and microfilament organization. To assess whether there were changes to the underlying cytoskeleton, we examined the effect of increasing concentrations of THC, CBD and CBN on the microtubules and microfilaments in CHO cells grown on glass coverslips. To localize microfilaments, the cells were simultaneously lysed and fixed with 0.1% glutaraldehyde, 2% paraformaldehyde and 0.5% Triton X-100 for 7 min, extracted in −20°C acetone for 2 min, rehydrated in phosphate buffered saline (PBS) at room temperature for 5 min, and incubated for 40 min at room temperature with 0.165 μM Bodipy-phallacidin (Molecular Probes, Junction City, OR) in PBS, pH 7.4. After a brief wash in PBS to remove any unbound probe, the coverslips were mounted onto slides with 50% glycerol, 1.5% n-propyl gallate in PBS, pH 8.3.

The typical distribution of microfilaments in untreated and 10 μM THC treated cells after 2 h is shown in Fig. 2. Prominent central stress fibers (bundles of actin microfilaments) extended throughout untreated (Fig. 2A) and vehicle-treated CHO cells. The overall morphology of the cells at 10 μM THC was similar to untreated controls. The number as well as the length of stress fibers observed in 10 μM THC-treated cells decreased compared to untreated controls (Fig. 2B). Prominent stress fibers were visible at the cell periphery, and many of the central stress fibers were either shorter or absent following THC treatment. In 10 μM CBD- and CBN-treated cells, there was little change in the morphology or distribution of stress fibers as compared to untreated controls. At 20 μM THC and 40 μM CBD and CBN, there was a noticeable increase in cell rounding and retraction of the cells, which was accompanied by a reduction in the number of central stress fibers relative to untreated controls.

Microtubules were observed in CHO cells by indirect immunofluorescence. The cells were lysed and fixed as described above, incubated in methanol for 4 min and acetone for 2 min

at −20°C. After rehydrating the cells in PBS, the cells were incubated with a primary antibody (monoclonal rat IgG specific for tubulin (MCA 77A, Cederlane Labs., Hornby, Ontario) diluted 1:100 in PBS, pH 7.4 for 30 min at 37°C followed by FITC conjugated goat anti-rat IgG (R40401, Cederlane Labs., Hornby, Ontario) diluted 1:25 in PBS, pH 7.4 for 30 min at 37°C. Any excess secondary was removed by 2 washes in PBS and the coverslips were mounted as described above.

In untreated (Fig. 2C) and vehicle control cells, the cytoplasmic microtubules formed an extensive network that appeared to originate from the microtubule organizing center located near the nucleus. Following exposure to 10 μM THC, there were distinct changes observed in the assembled microtubules (Fig. 2D). The microtubules generally appeared more granular and they were less distinct than in the untreated controls. Many globular vesicles around the nucleus which stained for tubulin were observed in the THC-treated cells. No changes were observed in the microtubule distribution following 10 μM CBD or CBN treatment as compared to the untreated controls; however, some changes, similar to 10 μM THC-treated cells, were observed in 40 μM CBD- and CBN-treated cells.

Influence of Cannabinoids on Biochemical Activities

Overview. Cannabinoids have been shown to suppress macromolecular synthesis in a number of cell systems by measuring the incorporation of radioactive precursors for DNA, RNA and protein. A THC-induced reduction in macromolecular synthesis in Tetrahymena has been reported (29,53). DNA, RNA, and protein synthesis were reduced in HeLa cells treated with THC, delta-8-THC, 11-OH-delta-9-THC and CBN (32,39) and DNA and RNA syntheses were depressed by THC in phytohemagglutinin-stimulated lymphocytes in vitro (1). Cannabinoid-induced suppression of macromolecular synthesis has also been shown in Lewis lung carcinoma cells by THC and delta-8-THC (9), cultured human fibroblasts by THC (4) and Leydig cells by THC, CBD and CBN (19). It has been suggested that the reduction of intracellular precursor pools may influence the amount of precursor available for incorporation into the acid-insoluble pool. Indeed, it has been shown, for example, that the decrease in macromolecular synthesis in HeLa cells reflected changes to the size of the radioactive precursor pool, which might suggest cannabinoid-induced effects on the permeability of the cell membrane (32).

The selective effects of cannabinoids on the expression of various genes have been described. In early studies, different RNA species, including heterogeneous high molecular weight RNA were reduced by THC (32 μM) in Tetrahymena (53). Studies on the specific expression in several human cultured cell lines indicated histone genes were preferentially inhibited (49). In rat hepatic microsomes, the amount of Cytochrome P450 UT-2 protein was selectively reduced following CBD treatment (36), and specific proteins normally expressed in response to bacterial immunomodulators in macrophages were suppressed by THC (8).

Influence of cannabinoids on tubulin and actin mRNA levels in CHO cells. It has been shown that the cellular architecture is dependent on the expression of cytoskeletal and cytoskeletal-associated proteins (2, 3, 13). The regulation of gene expression can occur at different levels, and may involve RNA transcription, RNA processing, RNA transport and/or stability of RNA transcripts. To determine if RNA transcripts for tubulin and actin mRNA were affected by cannabinoid treatment, we investigated the influence of cannabinoids on tubulin and actin messenger RNA levels by Northern blot hybridization. Total RNA was extracted from CHO cells treated at different concentrations of cannabinoids for 2 h, and an aliquot from each sample was fractionated on a 1.5% agarose-6% formaldehyde gel (14). The RNA was transferred to nitrocellulose filters, hybridized with a ^{32}P nick-translated cDNA probe for tubulin (PT25) or actin (PA72), graciously supplied by Dr. I. Ginzburg, Weismann Institute, Israel, and the relative amount of tubulin or actin mRNA levels were analyzed from autoradiographs by densitometry (54).

Tubulin mRNA levels were reduced 50 and 78% relative to untreated controls following a 2 h exposure to 5 and 10 μM THC, respectively (Fig. 3A). In contrast to the reduced levels of tubulin mRNA in the THC-treated cells, tubulin mRNA levels decreased no more than 13% following 5 and 10 μM CBD or CBN treatment. Actin mRNA levels were reduced 39 and 44% of untreated control values following a 2 h exposure to 5 and 10 μM THC, respectively (Fig. 3B). The level of actin mRNA remained unchanged up to 10 μM CBD and CBN treatment, followed by a 40% reduction at 20 μM.

The reduction in mRNA levels of tubulin and actin may have represented a general suppression in RNA synthesis; however, different lines of experimental evidence suggested that the reduction of mRNA levels was not a general cannabinoid-induced inhibition of RNA synthesis. Firstly, ^{3}H uridine incorporation into the acid insoluble fraction was investigated to determine if overall RNA synthesis was suppressed following THC treatment in CHO cells. Cells were pulsed with 5 μCi/ml ^{3}H uridine during the last hour of drug treatment in uridine-free medium. At the end of the pulse, the cells were chased with cold PBS containing uridine followed by two rinses with ice cold PBS. The cells were lysed and an equal amount from each sample was spotted in duplicate onto glass filter paper. One filter was counted to determine the total radioactive counts taken up by the cell. The other sample was used to determine the amount of label incorporated into the trichloroacetic acid (TCA) insoluble fraction. The effects of various THC concentrations on uridine incorporation into the acid insoluble fraction are shown in Fig. 4. In general, a dose-dependent increase in uridine incorporation occurred up to 10 μM THC, followed by a decrease. Since the effects of cannabinoids on cellular uptake can influence the amount of radioactive precursor available for incorporation, we indirectly measured the soluble pool of uridine precursor by subtracting the radioactive counts in the acid-insoluble fraction from the total counts (Fig. 4). The soluble uridine pool was reduced 20 and 10% relative to untreated controls at 0.1 and 10 μM THC. However, at 10 μM THC, the incorporation of radioactive precursor into the acid-insoluble fraction increased about 50%. Thus we propose that uridine incorporation into the acid-insoluble fraction was not influenced by the small fluctuations in the acid-soluble pool of uridine in CHO cells.

Secondly, a decrease of histone mRNA levels was reported between 10 and 40 μM THC in cultured human cells; however, a decrease in the levels of ribosomal RNAs was not observed (49). In our preliminary studies, a decrease in the levels of 18S ribosomal RNA relative to untreated controls was not observed after 10 μM THC treatment, as determined by Northern blot hybridization using a ^{32}P-labelled cloned human 18S sequence (graciously supplied by Dr. G. Stein, Univ. of Massachussetts Medical Centre).

Concluding Remarks

In our laboratory, we examined the influence of THC, CBD and CBN on the organization and changes in mRNA levels of cytoskeletal proteins in cultured CHO cells. In general, there

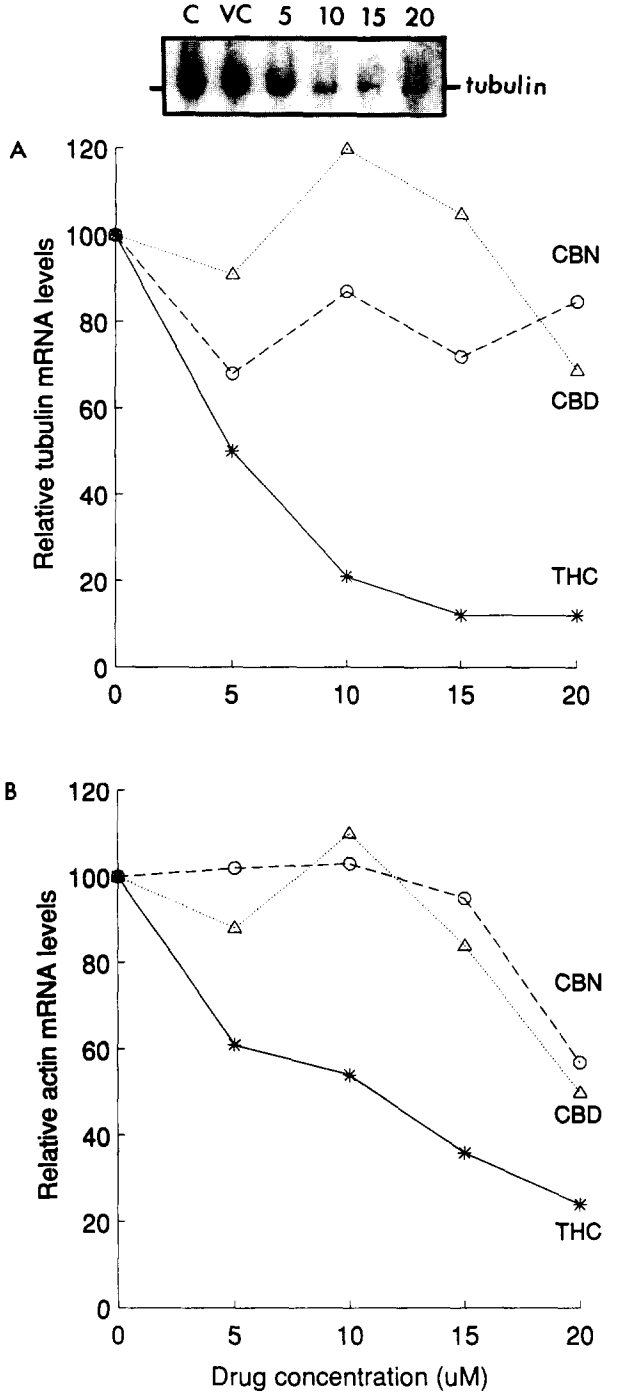

FIG. 3. Northern blot hybridization analysis of tubulin and actin mRNA levels in THC, CBD or CBN treated CHO cells for 2 h. Total RNA was phenol extracted from cells following treatment. A 10 μg aliquot of total RNA from each sample was fractionated by electrophoresis on a 1.5% agarose-6% formaldehyde gel, transferred to nitrocellulose and hybridized with a ^{32}P nick-translated tubulin (PT25) or actin (PA72) cDNA probe. Autoradiograms were analyzed by densitometry and the relative intensity of mRNA levels were determined and expressed relative to untreated controls. There was no difference in tubulin or actin mRNA levels between untreated and vehicle controls. The insert shows a representative autoradiogram of the region where the PT25 clone hybridized to the nitrocellulose filter. (A) Relative tubulin mRNA levels; (B) Relative actin mRNA levels.

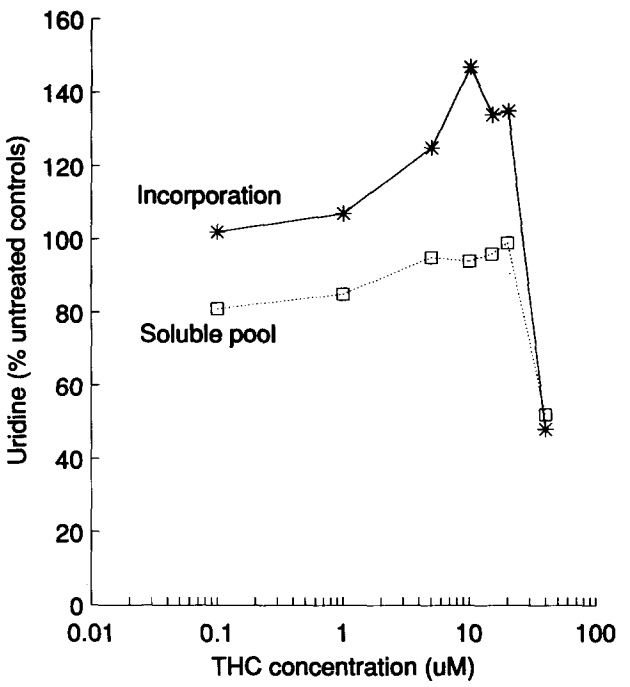

FIG. 4. Influence of THC on the incorporation of ^{3}H uridine into RNA. CHO cells grown in monolayer were pulsed during the last hour of drug treatment with 5 μCi/ml ^{3}H uridine. The incorporation of radioactivity into the acid-insoluble fraction was determined. The acid-soluble uridine fraction was estimated by subtracting the acid-insoluble fraction from the total cellular uptake of uridine for cells treated between 0.1 and 40 μM THC for 2 h. Data is expressed as a percentage of untreated controls. The vehicle had no effect on the incorporation of uridine as compared to untreated controls.

was a change in the cytoskeletal architecture and the mRNA levels of cytoskeletal proteins (tubulin and actin) in CHO cells following THC but not CBD or CBN treatment at concentrations which did not adversely affect cell proliferation. Stress fibers were reduced in number and length, and microtubules became fragmented in CHO cells treated with 10 μM THC for 2 h. Moreover, the levels of tubulin and actin mRNA transcripts were reduced.

The mechanism(s) by which THC alters the cytoskeletal architecture is unknown. THC may interact directly with the cytoskeletal elements or interfere with the process of assembly and disassembly. Preliminary reports have shown that THC influenced the in vitro assembly and disassembly of tubulin (40). THC may also affect the cytoskeleton indirectly by its effects on other cellular structures or other biochemical activities. Since the cytoskeleton is closely associated with cell membranes, THC interaction with the lipid bilayer or membrane bound enzyme systems, may adversely influence the cytoskeletal architecture. THC may also affect the permeability of the membrane to ions such as Ca^{2+}, an ion known to inhibit microtubule polymerization and disrupt actin microfilament assembly (37). THC may influence the organization of the cytoskeleton by affecting biochemical events involved in gene regulation. In our studies, the reduction in tubulin and actin mRNA levels may reflect cannabinoid-mediated effects on mRNA transcription, stability or processing of mRNA transcripts. Thus further studies on the cytoarchitecture and the expression of cytoskeletal proteins will be useful in assessing the effects of cannabinoids at the cellular and molecular levels.

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