BIOLOGY OF REPRODUCTION 27, 223–231 (1982)

In Vitro Effects of Cannabinoids on Follicular Function in the Rat[1]

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ABSTRACT

Δ^1-Tetrahydrocannabinol (Δ^1-THC), the major psychoactive constituent of marihuana, was found to suppress the preovulatory surge of gonadotropins and thereby to prevent ovulation in rats, rabbits and rhesus monkeys. These studies suggested that the drug acts primarily on the hypothalamus to suppress luteinizing hormone releasing hormone (LHRH) secretion. The aim of the present study was to examine the direct effect of Δ^1-THC, the psychoactive constituent of marihuana and cannabidiol (CBD), one of its nonpsychoactive constituents, on preovulatory rat follicles in vitro. Both cannabinoids inhibited follicular steroidogenesis in a dose-dependent manner. Basal accumulation of progesterone (P), testosterone (T) and estradiol-17β (E_2) was reduced up to 60% by the highest doses examined (100–200 μM). The luteinizing hormone (LH)-stimulated increase in P and T was inhibited by 75–88% by the highest doses of both cannabinoids (50–200 μM), while E_2 accumulation was inhibited by only 40%. It appears that the inhibitory action of cannabinoids is exerted beyond LH binding and activation of adenylate cyclase and prior to pregnenolone formation in the gonadal steroidogenic pathway.

In addition to this anti-steroidogenic effect, both cannabinoids induced resumption of meiosis in follicle-enclosed oocytes cultured in hormone-free medium; 200 μM Δ^1-THC resulted in 80% maturation and CBD in 75%. It seems that the action of cannabinoids on rat follicles in vitro is unrelated to their psychotropic activity.

INTRODUCTION

Administration of Δ^1-tetrahydrocannabinol (Δ^1-THC) to rats on the day of proestrus resulted in suppression of the preovulatory surge of LH and blockage of ovulation (Nir et al., 1973). Suppression of plasma follicle-stimulating hormone (FSH) and prolactin by administration of Δ^1-THC to proestrous rats has also been reported (Ayalon et al., 1977). Administration of Δ^1-THC to rhesus monkeys during the follicular phase, resulted in prolonged cycles, absence of the midcycle LH surge and elevation of luteal progesterone (Asch et al., 1981). Similarly, in the rabbit, which is a reflex ovulator, a single precoital dose of Δ^1-THC blocked the postcoital LH surge and ovulation (Asch et al., 1979). Administration of LHRH to Δ^1-THC-treated rats and rhesus monkeys resulted in release of LH (Chakravarty et al., 1975, 1979; Ayalon et al., 1977; Smith et al., 1979). These results indicate a direct effect of cannabinoids at the hypothalamic level.

Results of several studies suggest that in addition to the central effect of the cannabinoids, they may also act directly on the gonadal level. Thus, Δ^1-THC inhibited ovarian prostaglandin production in the rat (Ayalon et al., 1977) and testicular steroidogenesis in the mouse (Burstein et al., 1979a,b; Dalterio et al., 1977, 1978).

The aim of this study was to test the direct effect of cannabinoids on preovulatory follicles in vitro. For this purpose, Δ^1-THC, the major psychoactive ingredient of marihuana, and cannabidiol (CBD), one of its nonpsychoactive ingredients were studied.

Accepted February 18, 1982.
Received December 28, 1981.

[1] Presented in part at the Annual Conference of the Society for the Study of Fertility, Edinburgh, July, 1981, Abstract 96.

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MATERIALS AND METHODS

Animals

Three- to 4-month-old Wistar-derived rats from the departmental colony were housed in air-conditioned rooms, illuminated between 0500 and 1900 h. Pelleted food and water were provided ad libitum. Proestrus animals which had shown at least 2 normal 4-day cycles, as determined by daily vaginal smears immediately before the start of the experiment, were used.

Culture of Follicles

Animals were killed by cervical dislocation between 0800 and 1200 h on the day of proestrus. Graafian follicles were explanted from the ovaries and cultured as previously described (Tsafriri et al., 1972). Three follicles were cultured in each dish.

Chemicals

The following chemicals were added to the culture medium as indicated: LH (0.1–1.0 μg/ml, NIH-LH-S 20) (3R, 4R) Δ^1-tetrahydrocannabinol (Δ^1-THC) according to Gaoni and Mechoulam (1971); (-) cannabidiol (CBD) natural (crystalline, prepared at the Department of Natural Products, School of Pharmacology, The Hebrew University, Jerusalem); 8-bromo-cAMP (1 mM, Sigma, St. Louis); pregnenolone (1 μg/ml, Makor Chemicals Co., Jerusalem); cholesterol (1 μg/ml, Merck, Darmstadt); testosterone (1 μg/ml, Ikapharm); and 3 isobutyl-1-methylxanthine (IBMX, 5.10^{-4} M, Sigma).

LH and 8-bromo-cAMP were dissolved in 0.9% (w/v) NaCl, and Δ^1-THC, CBD, pregnenolone, cholesterol and testosterone were dissolved in absolute ethanol before dilution in the medium. The maximal final concentration of ethanol was 1% and this was shown to have no effect on the parameters studied. Medium was collected after 6 h of culture, replaced by fresh medium and collected again after further incubation of 18 h or as indicated.

Steroid Assays

The amounts of progesterone, testosterone and estradiol-17β in the medium were determined by a radioimmunoassay (RIA) as described by Lindner and Bauminger (1974). Steroids were extracted from the medium with 8 vol ether and the ether extracts were evaporated under weak N_2 stream, then redissolved in absolute ethanol. Well-characterized and specific antisera against progesterone, testosterone and estradiol-17β were used (Lindner and Bauminger, 1974). The minimum sensitivities and intra- and inter-assay coefficients of variation were: progesterone, 15 pg/ml, 2% and 20%; testosterone, 30 pg/ml, 4% and 18%; estradiol, 20 pg/ml, 3% and 20%, respectively. Cannabinoids did not interfere with the RIA of any of the steroids assayed.

Prostaglandin Assay

Prostaglandin E (E_1 + E_2) was determined by RIA as described by Bauminger et al., 1973. The total prostaglandin E production was determined by homogenizing the follicles in their culture medium at the end of the incubation period.

Determination of cAMP

Total cAMP accumulation (both in follicles and in the incubation medium) was determined by a modification (Lamprecht et al., 1973) of the Gilman protein-binding assay (Gilman, 1970). The follicles were incubated with different substances in the presence of IBMX for 30 min, at which time maximal cAMP accumulation in response to the hormones was achieved.

Examination of Follicular Oocytes

At the end of the experiment the oocytes were examined by Nomarski interference contrast microscopy (Tsafriri et al., 1972). Resumption of oocyte maturation was recognized by the breakdown of the germinal vesicle and disappearance of the nucleolus.

TABLE 1. Effect of cannabinoids on accumulation of steroids in culture.

Cannabinoid (μM)	No. of determinations	Steroid accumulation (ng/foll. 24 h)		
		Progesterone	Testosterone	Estradiol-17β
–	15	7.4 ± 1.5	1.5 ± 0.2	18.5 ± 8.5
Δ^1-THC				
50	3	5.2 ± 0.5	–	–
100	6	1.5 ± 0.2[b]	0.7 ± 0.02[c]	8.4 ± 1.0[c]
200	6	3.2 ± 0.9[a]	0.5 ± 0.1[a]	8.8 ± 1.2[c]
CBD				
100	9	1.4 ± 0.2[c]	0.6 ± 0.02[b]	6.5 ± 0.5[c]
200	9	4.0 ± 0.4[a]	0.4 ± 0.1[c]	9.1 ± 0.6[b]

[a] $P<0.05$ vs. appropriate control.

[b] $P<0.01$ vs. appropriate control.

[c] $P<0.001$ vs. appropriate control.

Histological Procedures

Some of the follicles were fixed in Bouin's solution for light microscopy or Karnowsky's solution for electron microscopy, dehydrated, embedded in paraffin or Epon and cut at 1 or 5 μm. Sections for light microscopy were stained with hematoxylin and eosin or toluidine blue and examined microscopically. Sections for electron microscopy were treated with uranyl-acetate and lead citrate.

Statistical Analyses

Statistical significance was determined by Student's *t* test or chi-square distribution depending upon the nature of the experiment.

RESULTS

Follicular Sterodogenesis

Follicular steroidogenesis was affected similarly by cannabinoids whether incubation was carried out for 6 h (data not shown) or 24 h. Preovulatory follicles incubated in LH-free medium released predominantly estradiol-17β, whereas the release of progesterone and testosterone was low. Addition of cannabinoids to follicles reduced the production of testosterone, progesterone and estradiol-17β to a similar extent (Table 1). Addition of LH (0.1 μg/ml) to the culture medium increased the production of the 3 steroids. The inclusion of Δ^1-THC or CBD in the culture medium reduced the accumulation of progesterone and testosterone in a dose-dependent manner, whereas accumulation of estradiol-17β was only slightly reduced. Of the 2 cannabinoids, CBD was more potent in decreasing steroid production (Fig. 1).

Is the Effect of Cannabinoids Exerted Through the Adenylate Cyclase Complex?

To determine whether cannabinoids exert their suppressive effect on steroidogenesis by acting on the adenylate cyclase complex, the amount of cAMP produced after a 30 min stimulation with LH (1.0 μg/ml) was measured in the presence and absence of both cannabinoids. There was a slight reduction in the cAMP production which did not reach statistical significance ($P>0.05$) (Table 2).

Moreover, the stimulatory effect of 8-bromo-cAMP (1 mM) on steroid production by the explanted follicles was inhibited in the presence of cannabinoids (data not shown).

The Steroidogenic Steps Inhibited by Cannabinoids

Addition of cholesterol (1 μg/ml) or preg-

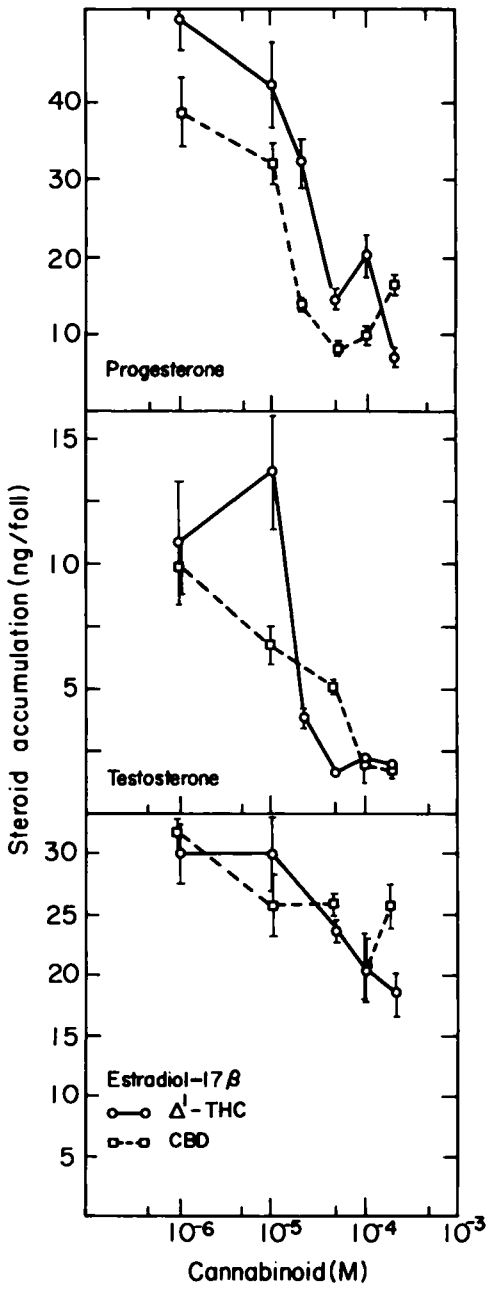

FIG. 1. Inhibition of LH-stimulated steroidogenesis by cannabinoids. Follicles were cultured for 24 h in the presence of LH (0.1 μg/ml) and the indicated concentration of cannabinoids. Steroids were assayed by RIA. The control values obtained in the presence of LH only were: progesterone, 52.6 ± 7.9 ng/foll.; testosterone, 16.0 ± 1.6 ng/foll.; and estradiol-17β, 33.5 ± 4.3 ng/foll.

TABLE 2. The effect of cannabinoids on follicular cAMP accumulation. LH (0.1 μg/ml) was added to all the cultures.

Cannabinoid (μM)	No. of determinations	cAMP (pmol/ foll. 30 min)	% of control
–	9	38.9 ± 1.3	100.00
Δ¹-THC			
25	3	36.6 ± 6.8	94.1
50	4	38.0 ± 0.9	97.1
100	7	33.1 ± 1.7	85.1
200	3	31.1 ± 3.5	80.1
CBD			
25	3	34.5 ± 2.6	88.9
50	4	40.9 ± 1.3	105.1
100	7	33.5 ± 1.8	86.4
200	3	31.6 ± 3.4	81.3

nenolone (1 μg/ml) to the culture medium resulted in a partial restoration of progesterone accumulation even in the presence of Δ^1-THC or CBD (Fig. 2).

Cannabinoids and Follicular Prostaglandin E Synthesis

Follicles were incubated for 5 h in a serum-free medium containing LH (1.0 μg/ml) and cannabinoids (50–100 μM). The results presented in Table 3 show that both cannabinoids suppressed prostaglandin E accumulation to the same extent.

The Effect of Cannabinoids on Maturation of Follicle-Enclosed Oocytes

Follicles cultured in hormone-free medium for 24 h usually contained a dictyate oocyte

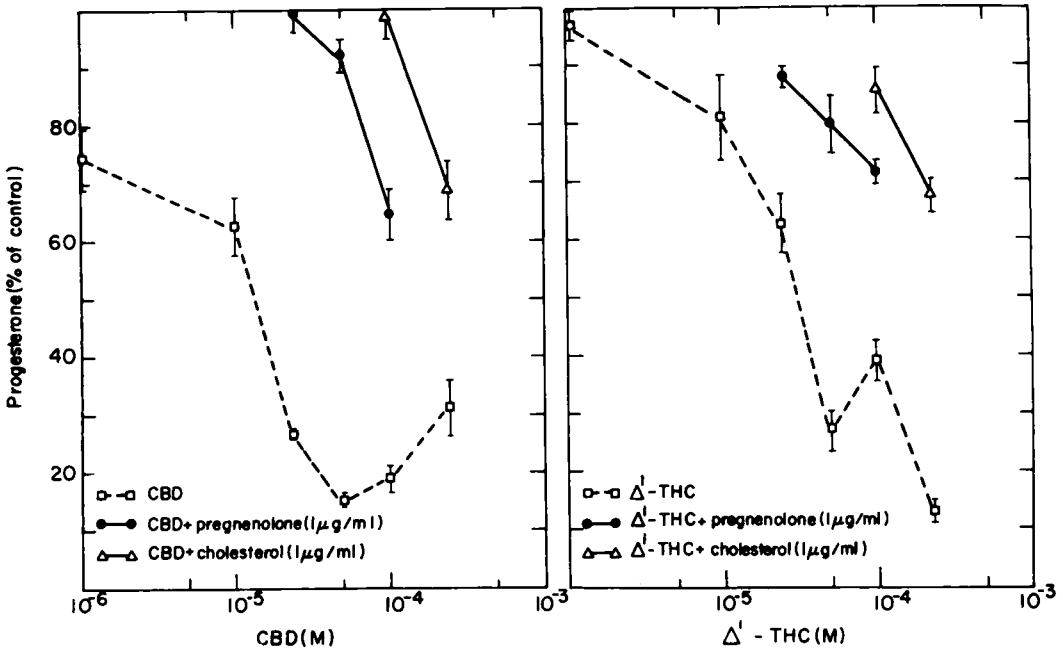

FIG. 2. Accumulation of progesterone in the presence of pregnenolone or cholesterol. The values obtained were compared to the control value of 52.6 ± 7.9 ng per foll. per 24 h.

TABLE 3. The effect of cannabinoids on accumulation of prostaglandin E (PGE) by Graafian follicles cultured for 5 h in serum-free medium. LH (1.0 μg/ml) was present in all the cultures.

Cannabinoids (μM)	No. of determinations	PGE pg/foll.	% of control
–	6	626.6 ± 71.5	
Δ¹-THC			
50	5	258.9 ± 57.3[b]	41.3
100	6	272.6 ± 60.7[b]	43.5
200	4	418.1 ± 34.3[a]	66.7
CBD			
50	6	324.0 ± 71.7[b]	51.7
100	6	280.4 ± 42.1[b]	44.3
200	5	280.7 ± 112.7[b]	44.8

[a] $P<0.005$ vs. control.

[b] $P<0.01$ vs. control.

and resumption of meiosis [germinal vesicle breakdown (GVB)] was seen only occasionally (4.7%). In the dictyate oocyte the cortical and perinuclear cytoplasm were rich in granular bodies (probably lysosomes, Ezzell and Szego, 1979) and mitochondria. The oolemma was studded with microvilli. The cumulus-granulosa cells were closely apposed to each other and their projections transversed the zona pellucida to form contact with the oolemma (Fig. 4 a and b). Addition of LH (0.1 μg/ml) to the culture medium induced GVB in 89% of the oocytes. The meiotic changes in the oocyte were accompanied by the detachment of cumulus-granulosa cells from each other and from the zona pellucida and withdrawal of their processes across the zona pellucida (Figs. 4 c and d). Addition of Δ^1-THC (100–200 μM) to hormone-free medium resulted in GVB in most of the follicle-enclosed oocytes, 41% ($P<0.001$) and 83% ($P<0.001$), respectively. Similarly, addition of CBD (100–200 μM) resulted in GVB in 70% ($P<0.001$) and 75% ($P<0.001$) of the oocytes, respectively (Fig. 3). However, meiotic maturation of oocytes induced by cannabinoids was not accompanied by changes in the cumulus-granulosa cells which normally occured when meiosis is induced by gonadotropins: the cumulus-granulosa cells remained closely packed and their projections across the zona pellucida did not retract (Fig. 4c).

When the time course of GVB of follicle enclosed oocytes in the presence of LH was compared to that induced by the cannabinoids, a delay of approximately 2 h in GVB was observed (Fig. 5).

DISCUSSION

This study showed that both Δ^1-THC, the major psychoactive ingredient, and CBD, a nonpsychoactive ingredient of marihuana, suppressed follicular steroidogenesis at a wide range of concentrations (Fig. 1). LH-stimulated accumulation of progesterone and testosterone was markedly reduced, whereas estradiol accumulation was only marginally affected.

A slight, but insignificant, reduction in the stimulation of follicular cAMP production by LH was observed when cannabinoids were

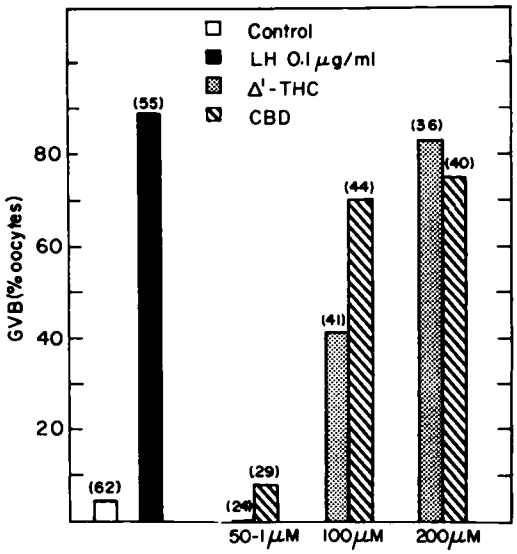

FIG. 3. Induction of oocyte maturation by cannabinoids. The number of oocytes examined is indicated in parenthesis.

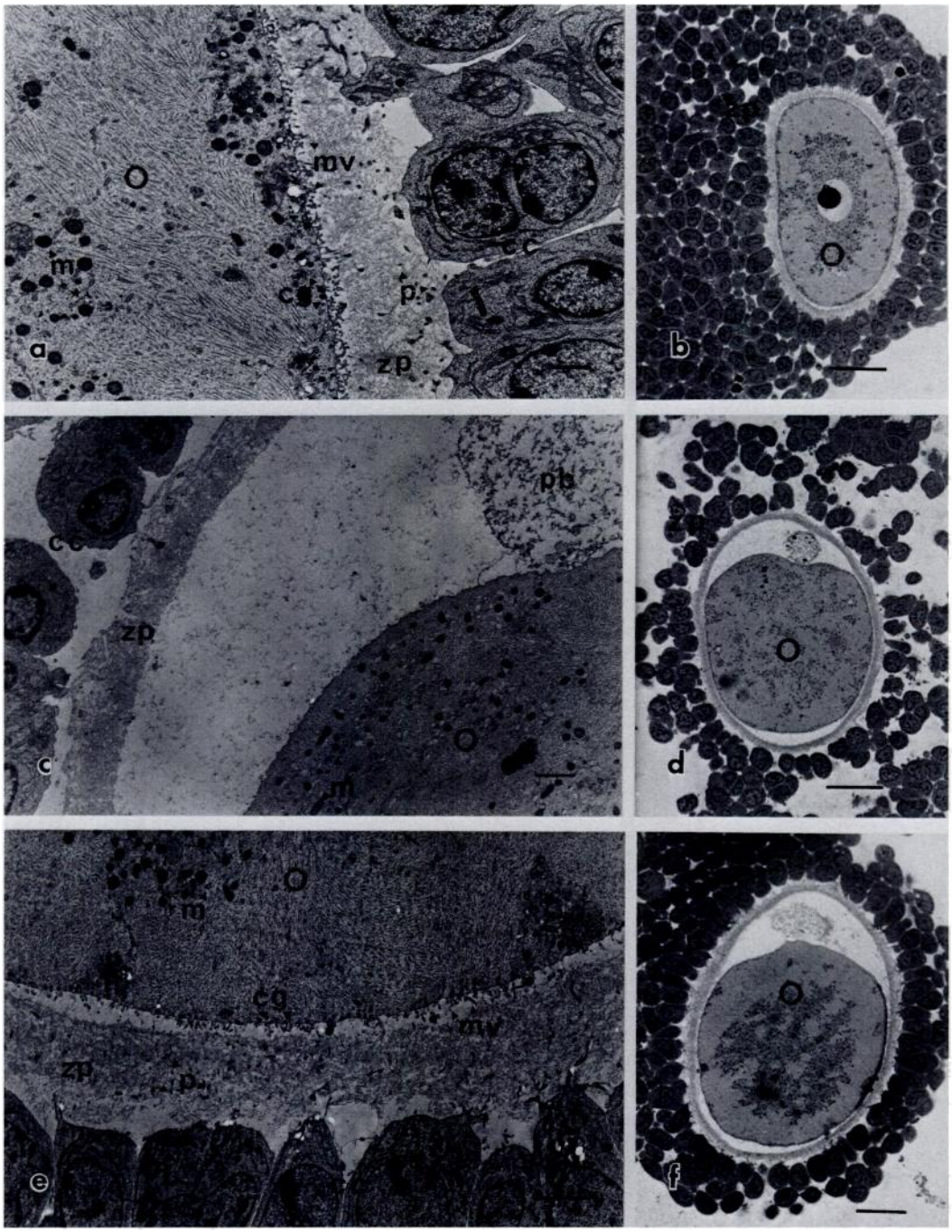

FIG. 4. Morphology of preovulatory Graafian follicles cultured for 24 h: (*a,b*) cultured in hormone-free medium; (*c,d*) with LH (0.1 μg/ml); (*e,f*) with CBD (200 μM). Electronmicrographs: *a,c,e* bar=2 μm. Micrographs: *b,d,f* bar=20 μm. Letters indicate: cumulus cells (*cc*), cortical granules (*cg*), mitochondria (*m*), microvilli (*mv*), oocyte (*o*), projections of cumulus cells (*p*), polar body (*pb*), and zona pellucida (*zp*).

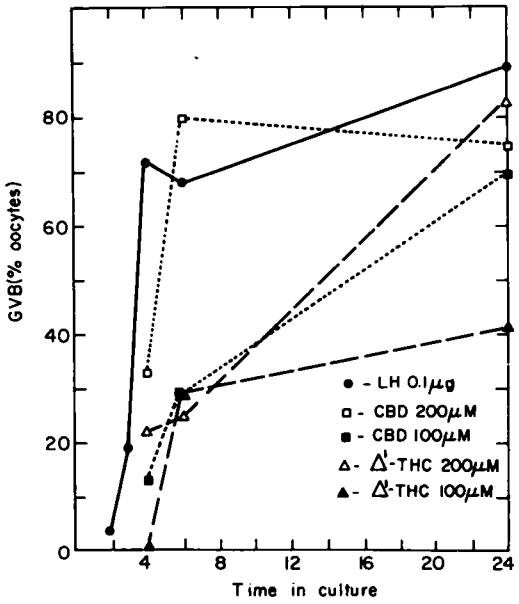

FIG. 5. Time course of germinal vesicle breakdown (*GVB*) in the presence of cannabinoids.

added to the medium. This inhibition was not observed at lower doses of cannabinoids which markedly inhibited follicular steroidogenesis. Therefore, it seems unlikely that the inhibitory effect of cannabinoids on steroidogenesis was at the adenylate cyclase level. This conclusion was further supported by the finding that addition of 8-bromo-cAMP to the culture medium was ineffective in reversing the anti-steroidogenic effect of cannabinoids. Thus it appears that cannabinoids suppress follicular steroidogenesis by acting distally to LH binding and activation of adenylate cyclase.

Addition of cholesterol to the culture medium resulted in partial restoration of follicular steroidogenesis as reflected by progesterone accumulation (Fig. 2). This may indicate that cannabinoids interfere with the release of cholesterol from its ester storage in lipid droplets, and thus limit the availability of the substrate for steroidogenesis. Since cannabinoids are known to affect membranal structures (Raz et al., 1972, 1973), including those of mitochondria, it is possible that cholesterol transport into the mitochondria is affected by the drug (Bino et al., 1971; Mahoney et al., 1972). Alteration of membrane transport by cannabinoids was observed in several models: uptake of nucleotides and of amino acids by human lymphocytes in culture (Nahas et al., 1977; Desoise et al., 1979); uptake of bioamines by brain synaptosomes (Hershkowitz et al., 1977, 1979); and uptake of fructose by human, rhesus monkey and rabbit sperm (Perez et al., 1981).

The enzymes responsible for the conversion of pregnenolone to progesterone do not seem to be influenced by cannabinoids since pregnenolone restored the steroidogenic activity of explanted follicles in the presence of cannabinoids. Similar results concerning the site of action of cannabinoids were reported by researchers working with mouse Leydig cells (Goldstein et al., 1977; Burstein et al., 1979b; Shoupe et al., 1980) with rat luteal cells (Burstein et al., 1979a); and with adrenal cells (Warner et al., 1977; Burstein et al., 1978).

The stimulatory effect of LH on follicular steroidogenesis is mediated by specific protein(s) which seems to be regulated at the transcription level (Tsafriri et al., 1973). Cumulative data from studies with several tissues indicate that macromolecular synthesis is affected by cannabinoids (Nahas et al., 1977; Desoise et al., 1979; Jakubovic et al., 1979 and others). However, inhibitory effect of cannabinoids on total follicular macromolecule synthesis was seen only well beyond the dose which inhibited follicular steroidogenesis (our unpublished observations). Nevertheless, this observation does not exclude a possible interference with the synthesis of a specific protein involved in steroidogenesis at lower concentrations of cannabinoids.

The study of Nir et al. (1973) clearly indicated the inhibition of the preovulatory surge of LH as the primary mechanism by which Δ^1-THC inhibits ovulation. However, treatment with LH, at a dose sufficient to bring about a complete ovulation in Nembutal-blocked rats (2.5 μg/rat), induced ovulation in only 40% of the animals treated with Δ^1-THC. In a later study, Δ^1-THC was shown to inhibit ovarian prostaglandin accumulation (Ayalon et al., 1977). The present results demonstrate a direct inhibitory effect of the two cannabinoids tested on follicular prostaglandin synthesis (Table 3). Since prostaglandins have an obligatory role in follicular rupture (reviewed by Lindner et al., 1974), the impeded ovarian response to LH observed by Nir et al. (1973) could be attributed to inhibition of follicular prostaglandin synthesis by Δ^1-THC.

High concentrations of cannabinoids mimicked the meiosis-inducing action of LH, but

the morphological changes in cumulus cell projections, which usually accompany oocyte maturation (Fig. 4) did not occur. It appears that action of cannabinoids on the resumption of meiosis is not specific and it may be related to the interaction of cannabinoids with membranal structures. Cannabinoids were found to interfere with divalent-cation ATPases in various tissues. Tsafriri and Bar-Ami (1978) demonstrated that a bivalent cationophore induced oocyte maturation. However, the meiotic changes in oocytes induced by the ionophore were delayed as compared to oocytes exposed to LH. A delay of 2 h in GVB was also noted in oocytes exposed to cannabinoids (Fig. 5). It is therefore reasonable to speculate that interference with bivalent ATPases and availability of bivalent cations underlies the effect of cannabinoids on ovum maturation. Further studies are needed in order to explore this hypothesis. In 25% of the rats in which ovulation was blocked by Δ^1-THC, the oocytes entrapped within the follicles were found to have matured (Nir et al., 1973). This partial maturation may be related to the direct effect of cannabinoids in ovum maturation observed in this study (Fig. 3).

This study clearly demonstrates a direct effect of the cannabinoids tested, Δ^1-THC and CBD, on follicular activity in vitro. Both inhibited follicular steroidogenesis and PGE_2 production and induced the resumption of meiosis in follicle-enclosed oocytes. The direct effect of the two cannabinoids on the ovary was unrelated to their psychotropic activity, thus suggesting that their effect on the ovary is probably exerted by a molecular site differing from that involved in the psychotropic activity of Δ^1-THC. It appears that the inhibitory action of cannabinoids is exerted beyond adenylate cyclase activation and at steroidogenic steps preceding pregnenolone synthesis. The precise mechanism of action of cannabinoids on preovulatory follicles remains to be determined.

ACKNOWLEDGMENTS

This investigation was supported by the Ford Foundation and the Rockefeller Foundation. We thank NIAMD for the supply of LH and Dr. R. Mechoulam for the supply of cannabinoids; Mr. S. Gordon and Mrs. A. Tsafriri for excellent technical assistance; Mrs. R. Levin for secretarial assistance. We are indebted to Dr. Y. Koch for his constructive criticism during the preparation of the manuscript.

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