Prostaglandins and Cannabis

XII. The Effect of Cannabinoid Structure on the Synthesis of Prostaglandins by Human Lung Fibroblasts

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Received June 25, 1982; Accepted September 22, 1982

SUMMARY

Earlier findings indicated that several other cannabinoids in addition to Δ^1-tetrahydrocannabinol (THC) were able to stimulate the synthesis of prostaglandins in cell culture systems. The present study was initiated to delineate the structural requirements for this effect within the cannabinoid series. Among the primary cannabinoids, we found that the trend was for more planar structures to show greater activity. In the case of THC metabolites, the order of activity was Δ^1-THC > 7-oxo-$\Delta^{1,6}$-THC > 7-OH-Δ^1-THC > 3″-OH-Δ^1-THC = 6β-OH-Δ^1-THC = 6α-OH-Δ^1-THC > $\Delta^{1,6}$-THC-7-oic acid. The latter sequence compares favorably with the available data on the behavioral assay in the rhesus monkey and the subjective "high" in humans. We also observed a good correlation between the release of arachidonic acid and the production of prostaglandin E, over a series of eight cannabinoids. This gives further support that the site of action in this effect is the elevation of activity of the phospholipase(s) responsible for supplying precursor arachidonic acid for prostaglandin synthesis.

INTRODUCTION

A number of reports over the last decade have prompted us to suggest that many cannabinoid effects are best understood by mechanisms involving changes in arachidonic acid metabolism (1). These changes could, in turn, result in alterations of the physiological levels of various prostaglandins, thromboxane A_2, prostacyclin, the leukotrienes, and other oxygenated products—many of which have potent biological activities (2).

One of the major regulatory enzymes controlling the synthesis of these substances is phospholipase A_2, which releases free arachidonic acid for the subsequent cascade of reactions leading to the physiological mediators (2). An alternative pathway resulting in the release of free arachidonate has recently been suggested as a source of precursor for the synthesis of prostaglandins and similar compounds (2). This involves the action of phospholipase C on phosphatidylinositol to give a diglyceride which is then further hydrolyzed to yield monoglyceride and free arachidonic acid.

Previous work in our laboratory has implicated the release of arachidonic acid by several cannabinoids as being responsible for the stimulatory effects of these cannabinoids on PG[2] synthesis (3–5). In this report, we have sought to demonstrate that there is, in fact, a direct link between the release effect and the elevation of PG levels. We also were interested in determining whether there is any obvious relationship between cannabinoid structure (Fig. 1) and stimulatory activity. We have continued to use human lung fibroblasts in monolayer culture as our model system since this system has proved to be suitable for such experiments in the past (4, 5).

MATERIALS AND METHODS

Chemicals. BSA, Fraction V, was purchased from Sigma Chemical Company (St. Louis, Mo.). [1-^{14}C]arachidonic acid (specific activity 52.7 mCi/mmole) and [^{3}H] PGE_2 (specific activity 165 Ci/mmole) were purchased from New England Nuclear Corporation (Boston, Mass.) and purified by thin-layer chromatography. PGE_2 was a gift from The Upjohn Company (Kalamazoo, Mich.). The cannabinoids were supplied by the National Institute on Drug Abuse, and their purity was monitored by gas chromatography and found to be greater than 95% pure. PGE antiserum was kindly donated by Dr. R. Skarnes, Worcester Foundation (Shrewsbury, Mass.). Its cross-reactivities with other prostaglandins were as fol-

This research was supported by Grants DA 02043 and DA 02052 from the National Institute on Drug Abuse.

[1] Recipient of Research Scientist Award DA 0043 from the National Institute on Drug Abuse.

[2] The abbreviations used are: PG, prostaglandin; BSA, bovine serum albumin; RIA, radioimmunoassay; MEM, minimal essential medium; TX, thromboxane; THC, tetrahydrocannabinol; CBN, cannabinol; CBCr, cannabichromene; CBG, cannabigerol; CBCy, cannabicyclol.

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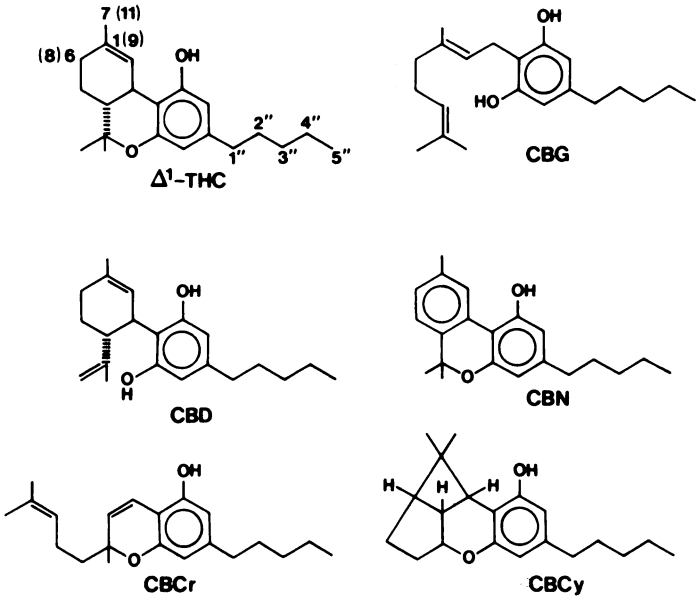

FIG. 1. *Structures of the primary cannabinoids*

The terpene numbering system is used in this report; the numbers in *parentheses* refer to the pyran system used by some authors.

lows: $PGF_{1\alpha}$, 4.0%; 6-keto-$PGF_{1\alpha}$, 1.0%; PGD_2, < 0.7%. Falcon disposable tubes were used throughout the RIA procedures.

Cells. WI-38 human lung fibroblast cells were obtained from Dr. L. Kelly (University of Massachusetts Medical School) and had been seeded from stock originally obtained from Dr. L. Hayflick (University of Florida). The cells were grown to confluence (3.1×10^5 cells/35-mm dish) in MEM containing 10% serum as described previously (6).

Incubation with cannabinoids. Serum-free WI-38 cells were incubated for 60 min under standard conditions (5) with 1 ml of MEM (pH 7.4), containing 0.1% BSA and cannabinoid or its vehicle (10 μl of ethanol). The media were harvested and the PGE concentration was determined by RIA as described previously (5). The results were expressed as nanograms of PGE per milliliter of culture medium; data storage and processing were carried out with the aid of an Apple II computer.

Labeling of cells. The WI-38 cells (approximately 3×10^5 cells/dish) were incubated with [1-^{14}C]arachidonic acid (1×10^5 dpm) in 1 ml of serum-free MEM for 60 min. The labeled cells were washed free of unreacted fatty acid with MEM. Under these conditions more than 35% of the incorporated radioactivity was present as phospholipids (4).

Drug treatment of the labeled cells. Labeled cells were incubated at 37° for 60 min with the cannabinoid delivered in 10 μl of ethanol and added to 1 ml of MEM containing 0.1% BSA. The supernatant was separated and an aliquot was analyzed for total radioactivity. The samples were then acidified to pH 3 with 1 ml of 0.001 N sulfuric acid and extracted with ethyl acetate (8 ml) containing the appropriate carriers, evaporated to a small volume, and chromatographed on silica-gel thin-layer plates using chloroform/methanol/acetic acid (90:6:6). The chromatograms were then stained with phosphomolybdic acid, and the zones corresponding to PGE_2 and TXB_2 were removed and assayed for ^{14}C by liquid scintillation counting.

Cell viability. Cell viability was determined by a modification of the DNA measurement method of Setaro and Morley (7). The monolayers were washed twice with 1 ml of MEM to remove nonadhering cells and then with cold trichloroacetic acid and potassium acetate. The DNA was extracted with 1 ml of 1 N perchloric acid per plate. Using microcuvettes and a Unicam SP 1800 spectrophotometer, the absorbance of these extracts at 260 nm was read against that of 1 N perchloric acid as the reference standard. In preliminary studies, our data have shown that the relationship between cell number and absorbance is linear. Calculating the percentage absorbance of the experimental plates as compared with the control plates will also give the percentage of viable cells.

RESULTS

On the basis of a report by Kelly and Butcher (8) and our own observations, we anticipated that high doses of cannabinoids would be toxic to WI-38 fibroblasts. In order to be able to select an appropriate series of doses for this project, we carried out a study of the effects of several cannabinoids on cell viability over a wide range of drug concentrations. Our measure of viability was based on DNA content as determined by a spectrophotometric procedure (7). Figure 2 compares the effects of CBN, CBD, Δ¹-THC, and 7-OH-Δ¹-THC on viability at doses of 0.8–32 μM. CBN and CBD had little effect until levels above 8 μM were reached, after which there was a rapid drop in cell survival. Δ¹-THC was slightly more toxic than CBN and CBD, showing an effect after 3.2 μM whereas 7-OH-Δ¹-THC, its major metabolite, had no significant effect on cell viability over the entire concentration range.

Another parameter to be established in defining our model was the time at which to test the effect. Under the conditions used, the cells reached confluence in about 4 days after plating. Since the drug response might have some dependence on cell density as well as the number of cells per plate, we measured the PGE elevation produced by 8 μM Δ¹-THC at several intervals after plating. Table 1 gives the results of this study, which had one unexpected feature. There was a steady rise in the stimulatory action of THC for the first 3 days, as anticipated; however, on day 4 the level dropped to a value between that of day 2 and day 3 and remained so through day 8. We decided on this basis to conduct all future experiments with 4-day-old cultures.

We next compared the stimulatory actions on PG synthesis of the six primary cannabinoids which were available to us, namely CBD, CBN, Δ¹-THC, CBCr, CBG, and CBCy. All of these cannabinoids were tested at a dose of 8 μM on the same batch of fibroblasts at the same time to eliminate as many variables as possible. The results are shown in Table 2 and are expressed both as absolute values and relative to cell viability. This latter way of reporting the effects perhaps gives a more accurate comparison, since cell survival did show some variation between the drugs. On this basis the order of potency is CBD > CBN > Δ¹-THC > CBCr > CBG ≅ CBCy.

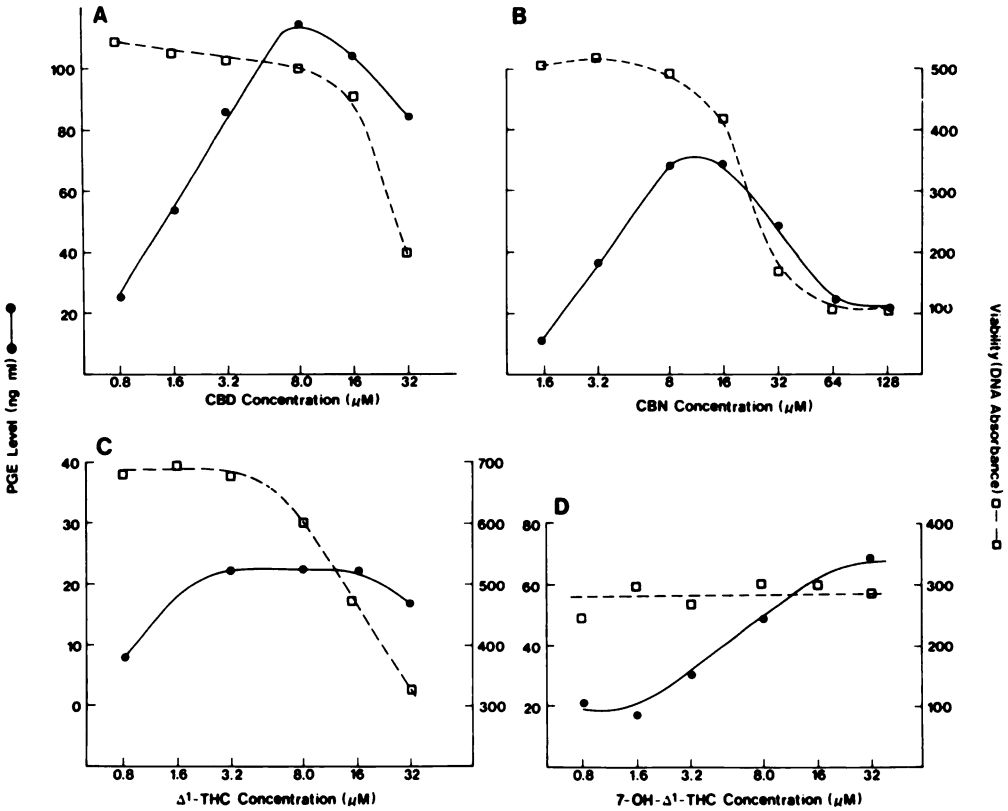

FIG. 2. *Cannabinoid effects on fibroblast viability compared with PGE production*

Cells were grown and incubated as described under Materials and Methods; the media were then analyzed for PGE content using the RIA procedure described above. Viability was measured as DNA content using the spectrophotometric procedure detailed under Materials and Methods.

In addition to the viability data, Fig. 2 shows dose-response curves for the effects CBD, CBN, Δ^1-THC, and 7-OH-Δ^1-THC on PGE levels. The results are given as the actual concentrations of PGE measured by the radioimmunoassay procedure. The variability in the maximal PGE levels is due in part to differences in the batches of cells; however, it is interesting to note that the primary cannabinoids generally showed steeper curves than the metabolites.

Figure 3 shows the dose-response curves for a series of metabolites of THC. These were obtained in individual experiments and are expressed as values relative to 8 μM Δ^1-THC, which was included in each experiment. Compensation was also made for decreases in cell viability at higher doses, so that responses reflect a constant cell number. Unmetabolized Δ^1-THC gave the strongest response; the 7-oxo-$\Delta^{1,6}$ derivative was slightly lower. Unfortunately, not all of the metabolites were available in the Δ^1 series. All of the monohydroxy metabolites were of lesser potency, and the 7-carboxy derivative showed little activity.

TABLE 1

Stimulatory action of 8 μM Δ^1-THC as a function of postplating time

Cells were seeded on 35-mm dishes at a density of about 1.5×10^5 cells/dish and grown as described under Materials and Methods.

Time	PGE[a]	
	Control	Δ^1-THC treated
days	*ng/ml*	*ng/ml*
1	1.65 ± 0.33	19.9 ± 1.3
2	0.17 ± 0.35	34.2 ± 2.7[b]
3	1.90 ± 0.63	57.4 ± 4.3
4	1.63 ± 0.20	39.0 ± 7.9[b]
7	4.17 ± 0.89	44.2 ± 3.9
8	4.92 ± 2.2	42.6 ± 2.5

[a] PGE was measured in the media by RIA using the charcoal-separation technique and the antiserum described under Materials and Methods. Values are means ± standard deviation.

[b] These values differed significantly from the day 3 THC-treated values ($p < 0.005$) as determined by Student's t-test ($N = 4$).

TABLE 2

Stimulatory effects of the primary cannabinoids on PGE production

Cannabinoid (8 μM)[a]	PGE[b]	PGE/viability[c]
	ng/ml	
CBD	60.3 ± 6.2	1.81
CBN	51.1 ± 2.0	1.52
Δ^1-THC	23.2 ± 1.2	1.24
CBCr	24.7 ± 1.6	0.98
CBG	18.7 ± 1.0	0.79
CBCy	19.4 ± 1.3	0.70

[a] The cannabinoids were added from stock solutions in 95% ethanol. A 10-μl volume was used which had been shown to have a negligible effect on PGE synthesis.

[b] As in Table 1. Values are means ± standard deviation.

[c] Relative values of PGE concentrations corrected for the number of surviving cells. Viability was determined by measuring cellular DNA as described under Materials and Methods.

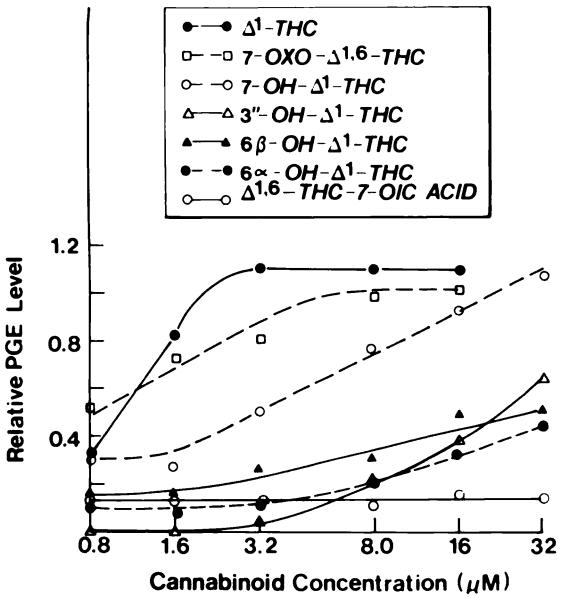

FIG. 3. *Effect of metabolism on cannabinoid stimulation of PGE synthesis in fibroblasts*

The cells were grown, incubated, and analyzed by RIA as described under Materials and Methods. PGE levels are expressed relative to the 8 μM Δ¹-THC control which was included in each experiment.

In order to be able to demonstrate that the ensuing elevation of PG levels was directly due to arachidonic acid liberation, we utilized the radiolabeled precursor approach described previously (4, 5). In the present report we have utilized this method using CBD as the agonist, whereas earlier experiments were conducted with Δ¹-THC. This was done to ensure the generality of the product distribution for all cannabinoids. Table 3 shows the thin-layer chromatographic distribution of radiolabeled products in the media from fibroblasts whose lipid pools contained esterified [^{14}C]arachidonic acid. Following CBD stimulation, both labeled PGE_2 and TXB_2 were significantly increased as compared with the products from vehicle-treated cells.

TABLE 3

Product composition from [^{14}C]arachidonate-labeled fibroblasts

Cells were grown and labeled as described under Materials and Methods. About 80% of the added radioactivity was incorporated, and 4.3% of this was released into the medium under control conditions; no BSA was present in the media.

TLC zone[a]	Control[b]	8 μM CBD[b]
	% total dpm	*% total dpm*
1 (origin)	4.6 ± 2.0	5.0 ± 2.3
2	9.8 ± 1.9	4.0 ± 1.0[c]
3 (TXB_2)	5.9 ± 0.7	7.4 ± 0.3[c]
4 (PGE_2)	19.0 ± 2.0	38.0 ± 2.0[c]
5	11.0 ± 1.0	6.7 ± 0.4[c]
6	9.9 ± 1.1	4.9 ± 0.2[c]
7 (PGA_2)	12.0 ± 2.0	6.6 ± 2.2[c]
8	11.8 ± 1.0	12.0 ± 2.0
9 (arachidonic acid)	10.0 ± 1.0	9.6 ± 1.2
10 (front)	5.1 ± 0.8	5.8 ± 0.9

[a] Thin-layer chromatography (TLC) was carried out as described under Materials and Methods. Zones were removed according to the location of the standards where possible. The remaining area was divided into equidistant zones.

[b] Values are means ± standard deviation as recovered from TLC. The control treated cells received 10 μl of ethanol.

[c] Significantly different ($p < 0.05$) from controls as determined by Student's *t*-test ($N = 3$).

The above experiment was conducted without BSA in the medium, which has been shown by others to have a profound effect on the extracellular levels of free fatty acids (9). Table 4 shows the effectiveness of BSA in "unmasking" the release of [^{14}C]arachidonate in our model. The data are expressed as a percentage of the radiolabel content of the cells, since the content varied somewhat between experiments. It is interesting to note that, although there was a 12-fold increase in released arachidonate, the effect of BSA on PGE_2 release was minimal.

A comparison of the release of [^{14}C]arachidonic acid with the synthesis of [^{14}C]PGE_2 is shown in Fig. 4A. A series of nine cannabinoids and metabolites was tested at a concentration of 8 μM, using the same preparation of cells. The values of each measurement are expressed relative to a value of 1.0 for Δ¹-THC. All of the substances (with the exception of CBCy) showed a clear correlation between the release of arachidonate and the synthesis of prostaglandin. The correlation coefficient was $R = 0.878$; by excluding CBCy this could be improved to $R = 0.975$.

A second comparison was made between [^{14}C]arachidonate and PGE synthesis as measured by radioimmunoassay (Fig. 4B). In this instance only six cannabinoids were tested, and the results were again expressed relative to Δ¹-THC. A good correlation was obtained when CBCy was omitted ($R = 0.85$); however, inclusion of CBCy resulted in a poor correlation ($R = 0.60$).

DISCUSSION

The cannabinoids tested in this study showed a wide range of stimulatory activities on both the synthesis of prostaglandins and the release of arachidonic acid from fibroblasts in culture. Some generalizations regarding the relationships of structure to activity can be made, although—as in any study of this type—it is not possible to be too precise in defining structural requirements.

An examination of Table 2 suggests that, in comparing the primary cannabinoids, activity may be related to planarity of the molecule, among other factors. CBCy is the compound least able to assume a planar conformation and it is the least potent. At the other end of the list, CBN and CBD are the most planar structures, although on this basis CBN should be the most active. There is no

TABLE 4

Effect of BSA on the release of arachidonate by 8 μM CBD

Cells were grown and labeled as described under Materials and Methods. BSA (0.1%) was added where indicated.

Product[a]	− BSA		+ BSA	
	dpm ± SD	% dpm[b]	dpm ± SD	% dpm[b]
PGE_2	1360 ± 82	1.65	934 ± 144	1.17
Arachidonic acid	341 ± 33	0.42	3270 ± 722	4.10

[a] As in Table 3.

[b] Values are the fractions of radioactivity in this thin-layer chromatographic zone based on the amount of ^{14}C incorporated in the cells.

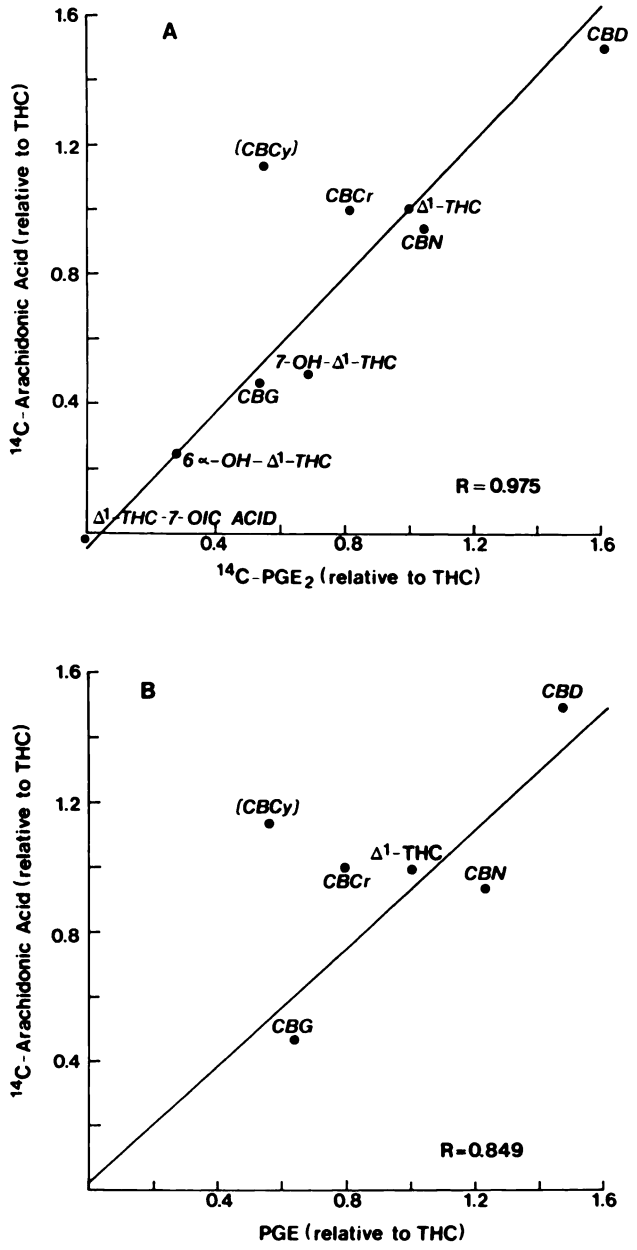

FIG. 4. *Comparison of arachidonic acid release and PGE synthesis in fibroblasts*

The release of [^{14}C]arachidonate is compared with the synthesis of [^{14}C]PGE_2 (A) or the endogenous synthesis of PGE (B). All cannabinoids were added at a concentration of 8 μM as described under Materials and Methods. The curves shown do not include the values for CBCy and resulted from the following equations: A, $y = 1.023x - 0.048$; B, $y = 0.939x + 0.019$.

obvious explanation for this discrepancy; it seems that CBD is better able to occupy whatever site in the cell is involved in this action.

With regard to the site of action, we have previously suggested that the cannabinoids may act on sites occupied by cholesterol (10). Since cholesterol is a relatively planar molecule, our present findings lend further support to this hypothesis. In this connection, it has been reported recently that platelets enriched in cholesterol become hypersensitive to aggregation; this was shown to be related to an increase in arachidonic acid release resulting in enhanced TXA_2 production (11).

In comparing the activities of those metabolites which were available to us, a second factor seems to have emerged. In general, the addition of oxygen to a cannabinoid decreases its potency as a stimulator of PGE synthesis (Fig. 3) as compared with the activities of the primary cannabinoids (Table 2). This finding suggests that the site of action is probably in a lipophilic region of the cell such as the plasma membrane. This is, in fact, where one would expect a lipase stimulator to exert its action.

Our main objective in this study was to provide additional evidence that the PG effect is due to cannabinoid action on a lipase (presumably phospholipase A_2). The close correlation between the release of [^{14}C]arachidonate and the synthesis of [^{14}C]PGE_2 which we found (Fig. 4A) provides strong support for this suggestion. It should be pointed out that it is necessary to have BSA present in the media during the drug treatment to trap the released arachidonic acid. There is no significant effect of BSA on PGE_2 synthesis (Table 4), so we believe that our data reflect an accurate measure of cannabinoid action on both processes.

One substance, CBCy, did not correlate well in this series. As was pointed out above, CBCy differs markedly in its molecular conformation from all of the others. Our findings suggest that it is relatively more effective as a lipase stimulator than is any substance in the rest of the series. A possible explanation is that CBCy can cause the release of arachidonate from cellular compartments which do not affect PG synthesis.

A possible weakness in using the radiolabeled-precursor approach is that the relevant cellular sources of arachidonate would not be labeled to the same extent as other lipid pools. Figure 4B compares the release of [^{14}C] arachidonate with the synthesis of PGE from endogenous arachidonate. Although data for only six cannabinoids were obtained, the pattern is similar to that from the measurement of [^{14}C]PGE_2 (Figure 4A). If CBCy is omitted, the correlation in this comparison is reasonably good. This would seem to imply that the labeling conditions utilized by us are appropriate for measuring effects on PG synthesis.

Our observation on the effect of Δ^1-THC as a function of cell growth (Table 1) may be of interest in studies of compounds other than cannabis. Taylor and Polgar (12) have recently reported a similar finding using human embryonic lung fibroblasts with bradykinin as the stimulator. They concluded that cell density per se was not a factor and that the rate of growth seemed to be related to responsiveness. Growing cells were less responsive, whereas quiescent cells produced many-fold higher levels of PGE_2 when stimulated by bradykinin. This was generally the pattern we observed with THC, except that we found a significant peak of activity at 3 days.

The relevance of our findings to the pharmacology of the cannabinoids poses an obvious question. In discussing this question, emphasis must be placed on what may be a well-known fact: the cannabinoids can exert a number of pharmacological actions which may or may not arise from a common mechanism. Since few structure-activity

studies have been reported on cannabinoid effects other than behavioral responses, at the present time we are restricted to this type of comparison.

An examination of relative potencies for the series of THC derivatives in Fig. 3 gives the following order of activity: Δ^1-THC > 7-oxo-$\Delta^{1,6}$-THC > 7-OH-Δ^1-THC > 3″-OH-Δ^1-THC = 6β-OH-Δ^1-THC = 6α-OH-Δ^1-THC > 7-COOH-$\Delta^{1,6}$-THC. This compares well with the relative activities reported for the behavioral assay in the rhesus monkey (13) and for the subjective "high" in humans (14, 15). It would be premature to conclude from this comparison that these *in vivo* effects are mediated by changes in PG synthesis. However, it is not unreasonable to suggest that the PG stimulatory action and the psychotropic responses are each initiated by similar molecular interactions such as drug alteration of membrane lipid conformation.

Other *in vivo* actions of Δ^1-THC may well require the stimulation of PG synthesis, of which there are two examples in the literature. The cataleptic effect in mice can be blocked by pretreatment with aspirin and indomethacin, and is restored by injection of PGE_2 (16). Moreover, mice deficient in polyunsaturated fatty acids exhibit a low cataleptic response which can be restored by the administration of arachidonic acid (17). Second, we have shown previously that the hypotensive action of Δ^1-THC in dogs can be greatly reduced by aspirin pretreatment (18). It would be of interest to determine whether inhibition of PG synthesis would reduce the psychotropic effect of THC in man.

ACKNOWLEDGMENT

We would like to thank Mr. John Cruz, Jr., of the Department of Biochemistry, for growing the cells used in this study.

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