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# EFFECTS OF $\Delta^9$-TETRAHYDROCANNABINOL ON GLUCAGON RECEPTOR COUPLING TO ADENYLATE CYCLASE IN RAT LIVER PLASMA MEMBRANES

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**Abstract**—Δ-Tetrahydrocannabinol ($\Delta^9$-THC), the principal psychoactive constituent of *Cannabis sativa*, was found to increase glucagon activation of liver plasma membrane adenylate cyclase. In the presence of 30 μM $\Delta^9$-THC, the $EC_{50}$ for glucagon was decreased by 60% from 7.6 nM to 3.1 nM. 11-OH-$\Delta^9$-THC, a psychoactive metabolite of $\Delta^9$-THC, also increased glucagon activation of adenylate cyclase while two cannabinoids without marihuana-like psychoactive potency, cannabinol and cannabidiol, did not. At 30 μM, $\Delta^9$-THC either slightly decreased or had no effect on the activation of adenylate cyclase by GTP, Gpp(NH)p, fluoride ion, forskolin or ATP alone. $\Delta^9$-THC had no effect on the binding of [$^{125}$I] glucagon to liver plasma membranes. Arrhenius plots demonstrated that $\Delta^9$-THC and 11-OH-$\Delta^9$-THC, but not CBD, decreased the activation energy above the break temperature. Therefore, $\Delta^9$-THC increased the coupling of the glucagon receptor to adenylate cyclase apparently by removing a constraint on receptor-$N_s$ coupling.

$\Delta^9$-Tetrahydrocannabinol ($\Delta^9$-THC) was isolated from *Cannabis sativa* and structurally identified by Mechoulam and coworkers [1] over twenty years ago, yet little progress has been made in determining the mechanism(s) of its action. $\Delta^9$-THC has effects on a wide variety of membrane-associated proteins which has led several investigators to propose a membrane perturbant mechanism for its action [2, 3]. In support of this hypothesis, the cannabinoids are extremely lipophilic [4] and have been shown to perturb lipid organization in the acyl chain regions of brain synaptic plasma membranes [5] as well as phospholipid bilayers [2, 5, 6].

Adenylate cyclase (EC 4.6.1.1) is a key regulatory enzyme which mediates the actions of many hormones and neurotransmitters by changing the intracellular concentration of cyclic AMP. Hormones and neurotransmitters regulate enzyme activity through binding to cell surface receptors and modulating the activity of membrane-associated guanine nucleotide regulatory proteins [7]. The activity of this intrinsic membrane system is sensitive to alterations in membrane fluidity [8, 9]. In particular, the influence of membrane physical properties on glucagon stimulation of adenylate cyclase in liver membranes has been well characterized [10]. In the studies reported here, we have exploited the sensitivity to membrane perturbation of glucagon stimulation of adenylate cyclase in order to study the interactions of the cannabinoids with biological membranes.

## MATERIALS AND METHODS

*Materials.* The cannabinoids were generously supplied by the National Institute on Drug Abuse and were solubilized using an emulphor- (G.A.F. Corp., New York, NY) ethanol vehicle [11]. Glucagon was a gift from Dr W. W. Bromer of Eli Lilly & Co., Indianapolis, IN. [$^3$H] cyclic AMP was obtained from Amersham Radiochemicals (Amersham, Bucks, U.K. or Arlington Heights, IL) and [$^{125}$I]-glucagon was purchased from New England Nuclear (Boston, MA). All other chemicals and biochemicals were obtained from usual commercial sources.

*Tissue preparation.* Rat liver plasma membranes were prepared from male Sprague–Dawley rats weighing 200–300 g by the method of Pilkis *et al.* [12]. The fraction which accumulated at the 48.2–45.5% sucrose interface was removed and stored until use at −70° in 0.25 M sucrose containing 3 mM imidazole–HCl (pH 7.4). Protein determinations were made using either the method of Warren *et al.* [13] or Bradford [14].

Isolated hepatocytes were prepared from fed 225–250 g male Sprague–Dawley rats and cells (3–5 mg dry weight/ml) were incubated as previously described [15]. ATP content of the isolated hepatocytes was determined in an $HClO_4$ extract using the luciferase method [16]. Only those cells with an ATP concentration greater than 8.7 nmol/mg dry weight were used.

*Adenylate cyclase assays.* Liver plasma membranes (0.05 mg protein/incubate) were added to incubation media containing (final concentrations): 25 mM triethanolamine HCl (pH 7.6), 1 mM EDTA, 5 mM $MgSO_4$, 10 mM theophylline and an ATP-regenerating system of 7.4 mg/ml creatinine phosphate and 1 mg/ml of a creatinine kinase–BSA mixture (1:4). Enzyme activity was initiated by the addition of ATP to a final concentration of 1.5 mM. Final

assay volume was 100 μl. After the appropriate incubation period, the reaction was terminated by placing the tubes into boiling water (>85°) for 3 min. The tubes were centrifuged and a 50 μl aliquot of the supernatant was taken for assay of cyclic AMP.

Adenylate cyclase activity in isolated hepatocytes was estimated by measuring cyclic AMP accumulation in response to $10^{-6}$ M glucagon. After preincubation for 20 min at 37°, cannabinoids were added and the incubation was continued for 10 min. Glucagon was added and after the desired length of time, the cell suspension was spun through bromododecane into an $HClO_4$ (0.62 M) and sucrose (0.25 M) mixture. The neutralized supernatant was assayed for cyclic AMP.

Cyclic AMP was measured using the competitive protein binding assay of Brown [17]. The cyclic AMP binding protein was isolated from bovine heart using the method of Rubin *et al.* through step 3 [18]. The standard curve contained all of the elements of the adenylate cyclase assay mixture except tissue.

[$^{125}$I] *glucagon binding assay.* Specific binding of [$^{125}$I] glucagon to liver plasma membranes was carried out as described previously [19]. Membranes (0.075 mg protein/incubate) were added to assay mixture containing (final concentrations): 25 mM triethanolamine–HCl (pH 7.2), 1 mM EDTA, 5 mM $MgSO_4$, 1 mM theophylline and 2.5% BSA. The total incubation volume was 0.15 ml. Specific binding was defined as the [$^{125}$I] glucagon displaced by $10^{-6}$ M unlabelled glucagon and accounted for more than 90% of the total binding. Incubations were carried out at 36° for 10 min. Bound and free [$^{125}$I] glucagon were separated by centrifugation of a 100 μl aliquot through 250 μl of washing buffer (10% sucrose, 40 mM triethanolamine, pH 7.2, and 2.5% BSA). The centrifuge tubes were frozen in an acetone/dry ice mixture, the tips were cut off and the radioactivity was counted. The equilibrium binding parameters were calculated from glucagon competition curves using the method proposed by Akera and Cheng [20]. Competition curves were constructed using nine concentrations of unlabelled glucagon from $10^{-10}$ to $10^{-6}$ M and 1 nM [$^{125}$I] glucagon. The parameters $K_D$ (dissociation constant) and $B_{max}$ (binding site density) were calculated using data pooled from six experiments.

*Data analysis.* Comparisons among treatment groups and controls were made using Dunnett's modification of the *t*-test [21]. Activation energies and breakpoints in Arrhenius plots were determined using a least squares fitting procedure as described previously [22]. $EC_{50}$ values of glucagon activation of adenylate cyclase were determined using regression analysis [21].

## RESULTS

Adenylate cyclase activity in liver plasma membranes was stimulated by glucagon with an $EC_{50}$ of approximately 10 nM. In the presence of $\Delta^9$-THC, stimulation of adenylate cyclase activity by $10^{-9}$ M glucagon was enhanced (Fig. 1). Statistically significant increases were seen at $\Delta^9$-THC concentrations of 10 μM and greater. However, activity in response to a supramaximal concentration of glucagon ($10^{-6}$ M) tended to be diminished by the same concentrations of $\Delta^9$-THC.

The concentration response curve for glucagon is shown in Fig. 2. $\Delta^9$-THC, at 30 μM, shifted the curve to the left and decreased the $EC_{50}$ value for glucagon by 60%. The maximal enzyme activity in response

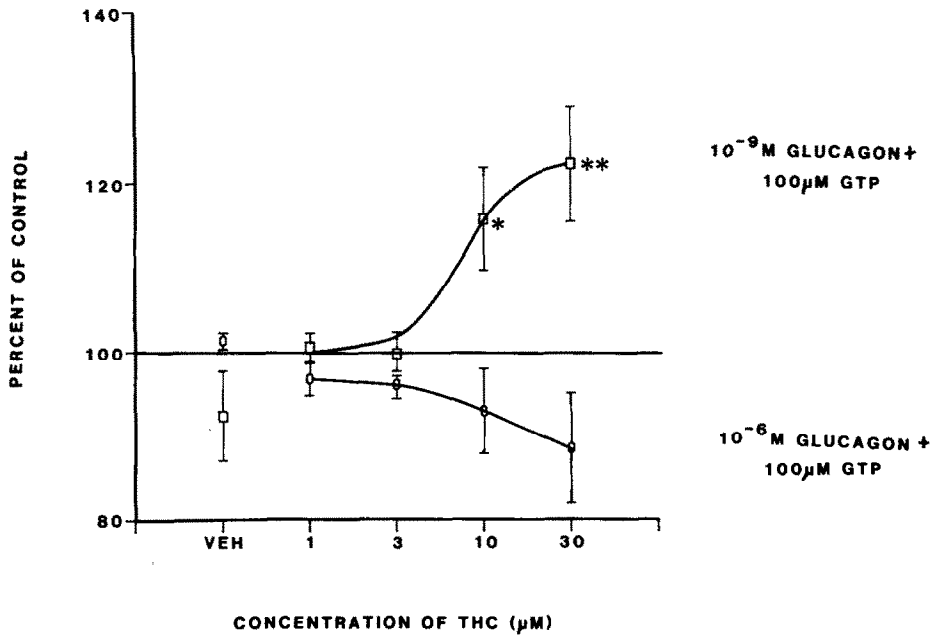


Fig. 1. Effect of $\Delta^9$-THC on glucagon stimulation of adenylate cyclase in liver plasma membranes. Each point shown is the mean value obtained from three experiments ± SEM. Control (untreated) activity for $10^{-9}$ M glucagon stimulation was 164 ± 8 pmol/min/mg protein. Control activity for $10^{-6}$ M glucagon stimulation was 459 ± 70 pmol/min/mg protein. "VEH" refers to vehicle equivalent to that administered with 30 μM $\Delta^9$-THC. * $P \leq 0.05$ compared to vehicle; ** $P \leq 0.025$ compared to vehicle.

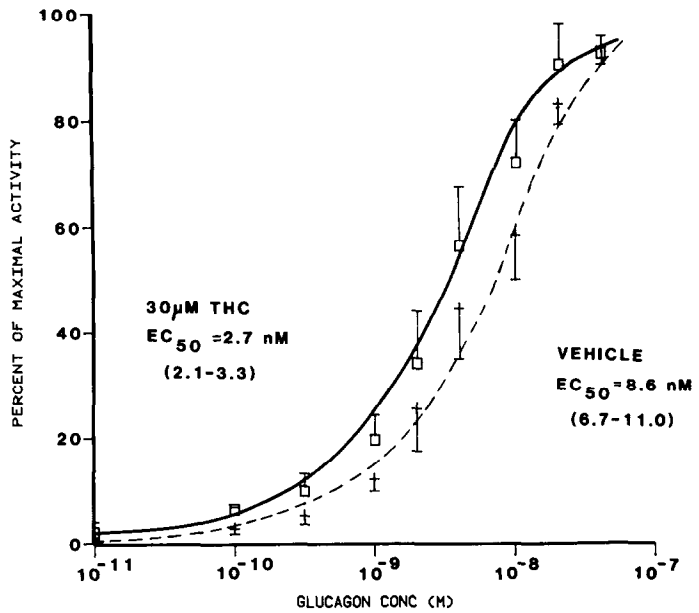


Fig. 2. Effect of $\Delta^9$-THC on glucagon concentration response curve in liver plasma membranes. Each point represents the mean of 3 experiments ± SEM. 100 $\mu$M GTP was present in all incubates. Data obtained in the presence of 30 $\mu$M $\Delta^9$-THC are represented by squares and solid line, vehicle is represented by plus signs and broken line. The maximal enzyme activities (activities in the presence of $10^{-6}$ M glucagon) are listed in Table 1. $EC_{50}$ values were obtained from log/logit plots of the data, values in the parentheses delineate the 95% confidence intervals for the $EC_{50}$ values.

to glucagon ($V_{max}$) was not significantly affected by this concentration of $\Delta^9$-THC (Table 1).

Activation of adenylate cyclase by glucagon is a complex sequence of events involving at least three intramembrane proteins. To assess the interactions of $\Delta^9$-THC with the enzyme itself, basal (ATP only) and forskolin stimulated activities were studied. At 30 $\mu$M, $\Delta^9$-THC had no effect on either basal or forskolin stimulation of adenylate cyclase (Table 2). Other concentrations of $\Delta^9$-THC between 1 $\mu$M and 50 $\mu$M were also without effect. Glucagon receptor activation of adenylate cyclase is mediated by an intramembrane, guanine nucleotide requiring protein, $N_s$. We assessed the effects of $\Delta^9$-THC on $N_s$ activation of adenylate cyclase using GTP, Gpp[NH]p (a nonhydrolyzable GTP analog) and $F^-$. At 30 $\mu$M, $\Delta^9$-THC had no effect on the adenylate cyclase activity in the presence of any of these agents (Table 2). Similarly, concentrations of $\Delta^9$-THC as low as 1 $\mu$M were without effect. These findings indicate that $\Delta^9$-THC does not act to stimulate adenylate cyclase directly or to potentiate $N_s$ activation of adenylate cyclase.

A possible mechanism for the potentiation of glucagon activation of adenylate cyclase by $\Delta^9$-THC is increased glucagon binding to its receptor. We determined the effect of $\Delta^9$-THC on the specific binding of [$^{125}$I] glucagon to liver plasma membranes.

Table 1. Effects of $\Delta^9$-THC on glucagon enzyme activation and bind parameters

| | Vehicle* | 30 $\mu$M $\Delta^9$-THC |
|---|---|---|
| Enzyme activation parameters† | | |
| $K_{act}$ (nM) | 7.6 | 3.1 |
| $V_{max}$ (pmol/min/mg prot) | 214 | 203 |
| Glucagon binding parameters‡ | | |
| $K_D$ (nM) | 109 | 109 |
| $B_{max}$ (pmol/mg protein) | 7.1 | 7.2 |
| Coupling factor | | |
| $K_D/K_{act}$ | 14 | 35 |

* Vehicle equivalent to that administered with 30 $\mu$M $\Delta^9$-THC.

† Parameters for glucagon stimulation of adenylate cyclase were derived from data shown in Fig. 2.

‡ Binding parameters were obtained from the combined data from 6 glucagon competition studies using 1 nM [$^{125}$I] glucagon and 9 concentrations of unlabelled glucagon from $10^{-10}$ to $10^{-6}$ M. Studies were done in the presence of 100 $\mu$M GTP.

Table 2. Effects of $\Delta^9$-THC on various stimulators of adenylate cyclase in liver membranes

| Stimulator | Activity (pmol/min/mg protein)* | |
|---|---|---|
| | Vehicle† | 30 $\mu$M $\Delta^9$-THC |
| Basal (N = 3) | 24 ± 4 | 22 ± 2 |
| 10 $\mu$M Forskolin (N = 4) | 562 ± 29 | 606 ± 31 |
| 100 $\mu$M GTP (N = 4) | 28 ± 2 | 30 ± 1 |
| 10 $\mu$M Gpp(NH)p (N = 3) | 182 ± 31 | 167 ± 20 |
| 15 mM NaF (N = 3) | 226 ± 16 | 200 ± 23 |

* Values are given as mean ± SEM. Enzyme substrate was 1.5 mM ATP.
† Vehicle equivalent to that administered with $\Delta^9$-THC.

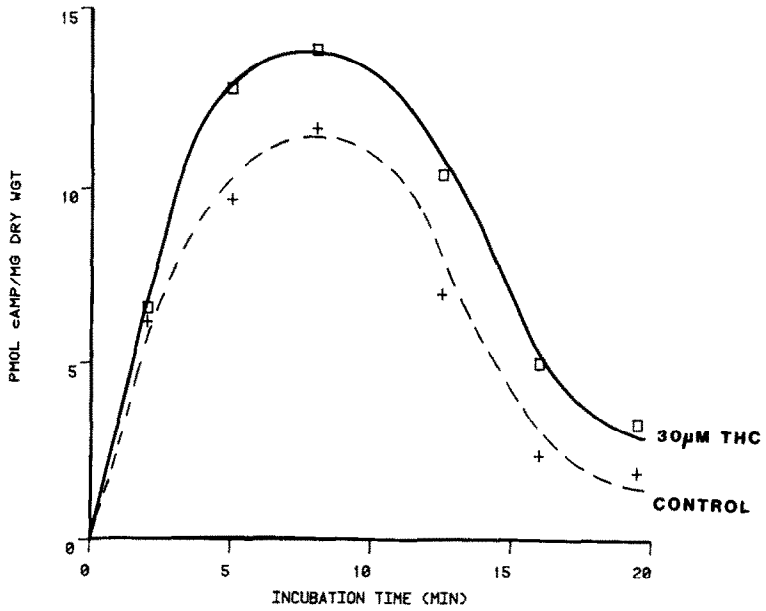


Fig. 3. Effect of $\Delta^9$-THC on glucagon stimulated accumulation of cyclic AMP in isolated hepatocytes. Data shown are the mean of 2 experiments done in triplicate. Glucagon concentration was $10^{-6}$ M. Vehicle had no effect on cyclic AMP accumulation. Data obtained in the presence of 30 $\mu$M $\Delta^9$-THC are represented by squares and solid line, control (untreated) data are represented by plus signs and broken line.

Table 3. Effect of cannabinoids on glucagon stimulation of adenylate cyclase in liver membranes

| Stimulator | Percent of vehicle* | | | |
|---|---|---|---|---|
| | $\Delta^9$-THC† | 11-OH-$\Delta^9$-THC | CBD | CBN |
| $10^{-9}$M glucagon (75 ± 2)‡ | 120 ± 5§ | 125 ± 10§ | 76 ± 8§ | 88 ± 15 |
| $10^{-9}$M glucagon + 100 $\mu$M GTP (119 ± 32)‡ | 137 ± 4§ | 136 ± 12§ | 76 ± 1§ | 108 ± 8 |
| $10^{-6}$M glucagon (280 ± 20)‡ | 93 ± 12 | 97 ± 10 | 77 ± 3§ | 85 ± 5§ |
| $10^{-6}$M glucagon + 100 $\mu$M GTP (464 ± 35)‡ | 89 ± 7 | 93 ± 2 | 75 ± 1§ | 77 ± 3§ |

* Vehicle equivalent to that administered with cannabinoids. Values shown are the mean of 3 experiments ± SEM.
† Cannabinoid concentrations were 30 $\mu$M.
‡ Enzyme activity in the presence of stimulator and vehicle (pmol/min/mg protein) ± SEM.
§ $P \leq 0.05$ compared to vehicle.

Table 4. Effect of cannabinoids on Arrhenius plots of glucagon stimulated adenylate cyclase activity

| | Break point* (°C) | Activation energy (kJ/mol) | |
|---|---|---|---|
| | | Above break | Below break |
| Vehicle† | 21.6 ± 0.7 | 46.0 ± 5.1 | 96.6 ± 4.4 |
| 30 μM $\Delta^9$-THC | 24.9 ± 1.9 | 21.2 ± 3.7‡ | 91.0 ± 3.5 |
| 30 μM 11-OH-$\Delta^9$-THC | 23.4 ± 0.6 | 26.6 ± 4.3‡ | 95.7 ± 7.0 |
| 30 μM CBD | 21.6 ± 1.4 | 34.4 ± 3.6 | 109.9 ± 5.8 |

* Break points and activation energies shown are the means of 4 experiments ± SEM. Initial rate measurements were made over a temperature range of 2–40° at 42 different temperatures. The break points and activation energies were derived using a least squares minimalization technique. Glucagon concentration was $10^{-6}$ M.

† Vehicle equivalent to that administered with the cannabinoids.

‡ $P < 0.025$ compared to vehicle.

Specific binding of [$^{125}$I] glucagon was saturable and displaceable by unlabelled glucagon. In the absence of GTP, the $IC_{50}$ for unlabelled glucagon in competition with 1 nM [$^{125}$I] glucagon was 1.4 nM and the Hill coefficient was 0.7. In the presence of 100 μM GTP, the $IC_{50}$ for unlabelled glucagon was increased to 110 nM and the Hill coefficient was increased to 1.4. These findings are in accord with those of previous investigators [23]. Concentrations of $\Delta^9$-THC from 1 μM to 50 μM had no effect on the binding of [$^{125}$I] glucagon to liver plasma membranes either in the absence or presence of GTP. At 30 μM $\Delta^9$-THC, neither the binding affinity nor binding site density of glucagon were affected (Table 1). As a result, the coupling factor, which is the ratio of the binding dissociation constant ($K_D$) and $K_{act}$ for adenylate cyclase, was increased by $\Delta^9$-THC, an indication that $\Delta^9$-THC increased the efficiency of glucagon activation of adenylate cyclase.

$\Delta^9$-THC also increased glucagon stimulation of cyclic AMP accumulation in isolated hepatocytes. At 30 μM, $\Delta^9$-THC increased hepatocyte cyclic AMP accumulation both in the absence (Fig. 3) and presence of the phosphodiesterase inhibitor isobutylmethylxanthine at a concentration (1 mM) known to fully inhibit cyclic AMP phosphodiesterase activity in hepatocytes [24] (data not shown). $\Delta^9$-THC had no effect on intracellular ATP content.

The effects of three other cannabinoids on glucagon activation of adenylate cyclase in liver plasma membranes were determined (Table 3). 11-OH-$\Delta^9$-THC is a major metabolite of $\Delta^9$-THC and is reported to be at least as potent as $\Delta^9$-THC in subjective studies of psychoactivity in humans [25]. Cannabidiol (CBD) and cannabinol (CBN) are constituents of cannabis which do not produce marihuana-like psychoactive effects in humans [25]. Like $\Delta^9$-THC, 11-OH-$\Delta^9$-THC significantly increased the stimulation produced by $10^{-9}$ M glucagon and had no effect on enzyme activity in the presence of $10^{-6}$ M glucagon. In contrast, neither CBD nor CBN increased glucagon stimulation of adenylate cyclase. In fact, CBD significantly decreased enzyme activity under all conditions studied and CBN significantly decreased the response to $10^{-6}$ M glucagon.

To explore whether the mechanism of action of $\Delta^9$-THC involved changes in the membrane bilayer physical properties, Arrhenius plot analysis of glucagon stimulation of adenylate cyclase was carried out. The data obtained were similar to those reported in earlier studies [22] except that the break points were consistently lower in the present study. Both $\Delta^9$-THC and 11-OH-$\Delta^9$-THC significantly reduced the activation energy above the break point but had no effect on the activation energies below the break (Table 4). Both tended to increase the temperature at which the break point occurred, although these changes were not statistically significant. Conversely, CBD had no effect on either the break point or the activation energies.

## DISCUSSION

The results reported in this communication demonstrate that $\Delta^9$-THC potentiated the activation of adenylate cyclase by glucagon in liver plasma membranes and increased the accumulation of cyclic AMP in isolated hepatocytes. A psychoactive metabolite of $\Delta^9$-THC, 11-OH-$\Delta^9$-THC, also increased glucagon stimulation of adenylate cyclase while two cannabinoids without marihuana-like psychoactive properties, CBD and CBN, did not. These findings suggest some degree of correlation between psychoactive potency and ability to enhance glucagon activation of adenylate cyclase.

Possible sites for this effect of $\Delta^9$-THC were explored. $\Delta^9$-THC had no effect on basal enzyme activity or forskolin, guanine nucleotide or fluoride ion stimulation of adenylate cyclase activity. Therefore, the potentiation produced by $\Delta^9$-THC is dependent upon the presence of glucagon and occurs at a step prior to $N_s$ activation. $\Delta^9$-THC does not increase the $V_{max}$ for glucagon stimulated adenylate cyclase, the increased enzyme activity is due to a decrease in the $EC_{50}$ (or $K_{act}$) for glucagon. $\Delta^9$-THC did not affect glucagon binding affinity which leads to the conclusion that its site of action is at the point of coupling between glucagon-occupied receptor and $N_s$. This is reflected in an almost 3-fold increase in the coupling factor, which describes the efficiency with which binding events lead to enzyme activation.

Previous studies of the interactions of the cannabinoids with adenylate cyclase have been somewhat contradictory. Dolby and Kleinsmith reported that micromolar concentrations of $\Delta^9$-THC enhanced both basal and norepinephrine stimulated

adenylate cyclase activities in mouse brain homogenates [26]. We have reported similar effects [27, 28], but found that the increase in basal activity could be antagonized by prostaglandin synthesis inhibitors. Kelly and Butcher [29] have demonstrated that the accumulation of cyclic AMP in human fibroblasts was increased by $\Delta^9$-THC, but that $\Delta^9$-THC decreased stimulation of adenylate cyclase by prostaglandin $E_1$ and epinephrine [30]. In a series of reports [31–33], Howlett has described and characterized an inhibitory effect of $\Delta^9$-THC on adenylate cyclase in neuroblastoma cell membranes. In those studies, low concentrations of $\Delta^9$-THC inhibited basal activity as well as stimulation by forskolin and several other agents. Inhibitory effects of $\Delta^9$-THC on adenylate cyclase activity in rat heart homogenates have also been reported [34]. Therefore, $\Delta^9$-THC clearly interacts with adenylate cyclase in a variety of tissues, but the direction of activity change appears to be dependent upon tissue type. Cannabinoid concentration may also play a role in the type of effect seen, although differences in methods of $\Delta^9$-THC solubilization make concentration comparisons between different investigators difficult to make.

Since the cannabinoids are perturbants of membrane bilayer ordering [2, 4, 5], we hypothesized that the effect of $\Delta^9$-THC on glucagon-stimulated adenylate cyclase was due to changes in membrane physical properties. The interactions of several other membrane active agents with this system have been studied using Arrhenius plot analyses and acyl chain probes to determine the relationship between membrane perturbation and enzyme activity. Agents such as benzyl alcohol [35], forskolin [36] and ethanol [36], all of which decrease membrane phospholipid ordering (increase "fluidity"), activate adenylate cyclase and decrease the Arrhenius break point for glucagon-stimulated activity. Conversely, cholesterol enrichment, which increases membrane ordering, inhibits enzyme activity and abolishes both the Arrhenius break point for enzyme activity and the lipid phase separation [37]. Therefore, if $\Delta^9$-THC increased enzyme activity as a result of increased bilayer fluidity, one would expect the Arrhenius break point to decrease. This is clearly not the case, so it is unlikely that increases in membrane disorder account for the cannabinoid effect. Furthermore, the effects of $\Delta^9$-THC were specific for glucagon stimulation, while benzyl alcohol [35] and ethanol [36] have been shown to increase fluoride and basal activities in addition to glucagon stimulation. Arrhenius studies demonstrated that $\Delta^9$-THC and 11-OH-$\Delta^9$-THC, but not CBD, decreased the activation energy above the break point. This suggests that the active cannabinoids relieve a constraint imposed upon the intramembrane coupling of the agonist-occupied receptor and $N_s$. The mechanism of this effect is unclear except that it does not appear to be due to decreased bilayer ordering.

These studies demonstrate that $\Delta^9$-THC interacts with glucagon stimulation of adenylate cyclase in liver plasma membranes in an indirect fashion. The effects of $\Delta^9$-THC differ from those of membrane bulk lipid fluidizers such as ethanol and benzyl alcohol, but could be a result of more specific perturbations of membrane constituents. The physiological importance of this effect is unknown. However, the enhancement of glucagon activation occurs in whole cells as well as isolated membranes. These studies offer further support for the hypothesis that cell membranes and their associated proteins are a significant site of cannabinoid action.

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