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Cannabinoid Inhibition of Adenylate Cyclase

Biochemistry of the Response in Neuroblastoma Cell Membranes

ALLYN C. HOWLETT

Department of Pharmacology, St. Louis University School of Medicine, St. Louis, Missouri 63104

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SUMMARY

The inhibition of adenylate cyclase activity by cannabimimetic compounds in a membrane fraction from cultured neuroblastoma cells has been examined. The inhibition was shown to be concentration-dependent over a nanomolar range for both Δ^9-tetrahydrocannabinol and its synthetic analog, desacetyllevonantradol. Inhibition was rapid and reversible. The cannabimimetic compounds caused a decrease in V_{max} of the enzyme, with no alteration in the K_m for substrate. The effects of these compounds were related to the ability of the enzyme to be regulated by divalent cations and guanine nucleotides. The inhibition was greatest at micromolar Mg^{2+} or Mn^{2+} concentrations and was abolished at <1 mM $MnCl_2$. In the hormone-stimulated state, the enzyme appeared to be regulated by one Mg^{2+} site. The addition of cannabimimetic or muscarinic cholinergic agents transformed the enzyme into one in which more complex regulation by divalent cations was observed. Half-maximal inhibition of adenylate cyclase was observed at 800 nM GTP for both cannabimimetic and muscarinic cholinergic agents. The substitution for GTP of a nonhydrolyzable analog resulted in activation of the enzyme and failure to respond to either class of inhibitory agents. If the Mg^{2+} concentration was reduced and exposure to the GTP analog was of short duration, inhibition by both cannabimimetic and muscarinic agents could be observed in the presence of forskolin. This study points to the similarities between the enzyme inhibition by cannabimimetic compounds and by muscarinic cholinergic compounds. It is inferred that the cannabimimetic compounds must act via regulatory mechanisms similar to those operating for receptor-mediated inhibition of adenylate cyclase.

INTRODUCTION

Our recent investigations have demonstrated that adenylate cyclase in plasma membranes from neuroblastoma cells is inhibited by low (<1 μM) concentrations of cannabimimetic drugs (1, 2). Δ^8- and Δ^9-THC,[1] the constituents of marihuana extract responsible for the neurologic and behavioral responses, and the nantradol class of synthetic bioactive analogs were effective inhibitors. The inhibition was specific for psychoactive cannabinoid compounds, since cannabinol and cannabidiol did not produce the response. Cannabimimetic inhibition was noncompetitive with respect to prostanoid or secretin stimulation of the enzyme. Basal and forskolin-activated enzymes were also inhibited. These results suggested that the cannabimimetic compounds may interact with adenylate cyclase in a manner similar to that by which hormones and neurotransmitters inhibit the enzyme.

The mechanism of action of cannabimimetic agents at the cellular level is not currently understood. One hypothesis is that these highly lipophilic compounds may intercalate into cell membranes and the resulting lipid perturbations may alter enzyme activities. Support for this hypothesis comes from membrane fluidity changes observed in cholesterol-lecithin liposomes (3) and more recently in synaptic plasma membranes (4). Alterations of a number of cell membrane functions have been attributed to the membrane effects of cannabinoid drugs (see references in Ref. 5). However, most of these latter observations have required concentrations at Δ^9-THC in excess of 10 μM and have not demonstrated pharmacologic specificity for psychoactive compounds.

A second hypothesis is that cannabimimetic agents interact with specific receptors or enzymes. This hypoth-

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[1] The abbreviations used are: THC, tetrahydrocannabinol; Da-*l*-Nan, desacetyllevonantradol; Ro 20-1724, 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone; Gpp(NH)p, guanosine 5′-(β,γ-imino)triphosphate; G_s, a triprotein complex (α_s, β, γ) that can interact with stimulatory receptors to ultimately increase adenylate cyclase activity; G_i, a triprotein complex (α_i, β, γ) that can interact with inhibitory receptors to ultimately decrease adenylate cyclase activity; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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esis is based on the observations that pharmacologic specificity and stereospecificity are observed for the psychoactive properties *in vivo* (5, 6).

The action of cannabimimetic compounds to inhibit adenylate cyclase activity *in vitro* provides us with an experimental system in which to test these hypotheses. Considerable work has been reported describing the inhibition of adenylate cyclase via specific membrane receptors for hormones (see Refs. 7–9 for review). The studies in this report examine the properties of adenylate cyclase that are influenced by cannabimimetic agents. Direct comparisons are made to the properties of inhibition via muscarinic cholinergic receptors.

EXPERIMENTAL PROCEDURES

Neuroblastoma membrane preparation. Cell line N18TG2, a clone derived from the murine C1300 spontaneous peripheral neuroblastoma (10), was thawed from frozen stocks (passage number 25) and maintained in culture 6–8 weeks. The cells were grown on 175-cm^2 culture flasks in Dulbecco's modified Eagle's/Ham's F-12 (1:1) medium containing 10% heat-inactivated calf serum, 50 IU/ml penicillin and 50 μg/ml streptomycin. Cells from confluent cultures were dissociated by gentle pipetting with a solution of 0.6 mM EDTA plus 10 mM Na HEPES, pH 7.4 in phosphate-buffered saline. Following a rinse in the same solution minus EDTA, cells were resuspended in 20 mM Na HEPES, pH 8.0, and 1 mM EDTA plus 2 mM $MgCl_2$ (HME buffer) and subjected to nitrogen cavitation disruption. The cell membranes from a 500 × *g* supernatant fraction were sedimented at 39,000 × *g* for 30 min. The precipitate was resuspended in 0.3 M sucrose in HME buffer plus 1 mM dithiothreitol, quick-frozen and stored at −80° until further purification. A membrane fraction further enriched in adenylate cyclase activity was obtained by sedimentation (28,000 rpm in a Beckman SW 28 rotor for 75 min) over a step gradient consisting of 10 ml each of 1.4, 1.0, and 0.6 M sucrose in HME buffer. The membranes aspirated from the denser two sucrose interfaces were diluted in HME buffer and sedimented at 35,000 rpm in a Beckman 35 rotor for 45 min. These membranes were resuspended to approximately 5 mg/ml in HME buffer containing 1 mM dithiothreitol, rapidly frozen in small aliquots, and stored at −80° until use. Protein was determined by the method of Bradford (11) using a γ-globulin standard.

Assay for adenylate cyclase. Rapidly thawed membranes were incubated in a 100-μl volume containing the following: 50 mM Na HEPES, pH 8.0, 1 mM EDTA, 5 mM $MgCl_2$, 100 μM GTP, 0.1 mM Ro 20-1724, 0.1 mM cyclic AMP, 10 nCi of cyclic [^{3}H]AMP, 0.1 mg/ml fatty acid-deficient bovine serum albumin, 10 μg/ml pyruvate kinase, 3.0 mM K^+ phosphoenolpyruvate, 0.5 mM ATP, and 0.5 μCi of [α-^{32}P]ATP. Modifications to this mixture are described in the figure legends. Unless otherwise stated, assays were run at 30° for 20 min. Cyclic [^{32}P]AMP was isolated according to Salomon *et al.* (12). Triplicate determinations were made for each data point, and the coefficient of variation was less than 10% for these experiments.

Because of the avidity with which cannabinoid compounds bind to plastic and glass surfaces, assays were conducted in 10 × 75 mm borosilicate glass tubes that had been silylated with bis(trimethylsilyl)trifluoroacetamide (Regisil RC-1) as recommended by Garrett and Hunt (13). Lang-Levy pipets and test tubes used for dilutions were similarly treated. Da-*l*-Nan and Δ^9-THC were made up as 30 and 100 mM stock solutions in absolute ethanol and stored at −80°. Initial dilutions to 1 mM were made in 50 mg/ml fatty acid-deficient bovine serum albumin (14) warmed to 30°. Subsequent dilutions were made in HME buffer containing 0.1 mg/ml fatty acid-deficient bovine serum albumin. This procedure, similar to that described by Perez-Reyes *et al.* (15), resulted in a clear microsuspension of these compounds. Other techniques of aqueous suspension in the absence of detergents resulted in a cloudy suspension that ultimately formed a viscous plaque at the meniscus. The aqueous solubility of Δ^9-THC has been reported to be 6 μM at the approximate ionic strength of the adenylate cyclase assay (13). Thus, insolubility during the assay procedure is not a problem after the initial dilutions have been made.

Determination of K_m for Mg-ATP. The K_m for substrate was determined by varying the concentration of Mg-ATP (5 to 200 μM) at several fixed concentrations of $MgCl_2$ (0.5 to 10 mM). The concentration of EDTA in the reaction was reduced to 80 μM, equivalent to the 80 μM $MgCl_2$ present with the membranes. All activities were assayed for 10 min in the presence of 100 μM GTP and 500 nM secretin. Double reciprocal plots were constructed from the data, and slopes and intercepts were determined by linear least squares regression analysis. Secondary replots of the slopes and intercepts were analyzed by linear regression to obtain the V_{max} and the K_m for Mg-ATP at infinite Mg^{2+}. Three sets of experiments were performed, using two different membrane preparations, and the data were analyzed statistically using Student's *t* test for paired comparisons.

Materials. [α-^{32}P]ATP and cyclic [^{3}H]AMP were purchased from New England Nuclear. Secretin was from Bachem and handled as previously described (16). Forskolin from Calbiochem was stored and diluted as described above for the cannabinoid compounds. Δ^9-THC was obtained from the National Institute of Drug Abuse. Da-*l*-Nan was a generous donation from Pfizer, and Ro 20-1724 was a gift from Hoffmann-La Roche.

RESULTS

Kinetics of adenylate cyclase inhibition by cannabimimetic agents. Inhibition of adenylate cyclase by Δ^9-THC and by Da-*l*-Nan is shown in Table 1 to be in the nanomolar range. Da-*l*-Nan inhibited at less than 2 nM and approached the maximal effect at 200 nM. The inhibition by Δ^9-THC was less potent, beginning at greater than 20 nM and reaching the maximal response at about 2 μM. These results are similar to those observed previously using somewhat different conditions (2). In that report, a reversal of the inhibition was demonstrated at cannabinoid drug concentrations exceeding 3 to 10 μM. It was postulated that if the stimulatory portion of the biphasic response began at a concentration less than that required for the maximal inhibitory response, then Δ^9-THC would appear to inhibit adenylate cyclase less effectively. Drug concentrations of 3 μM or less are used in the experiments in the present study, in order to maximize the inhibition observed.

Inhibition of adenylate cyclase by cannabimimetic agents could be discerned within 1 min following addition of the drugs. No lag period was apparent whether Δ^9-THC and Da-*l*-Nan were added at the start of the reac-

TABLE 1

Concentration-effect relationship for cannabimimetic inhibition of adenylate cyclase

Secretin was present at 500 nM and cannabimimetic drugs were at the indicated concentrations. Numbers in parentheses are the per cent inhibition compared with the activity for secretin alone.

	Adenylate cyclase	
	Δ^9-THC	Da-*l*-Nan
	pmol/min/mg	
Secretin	229	230
Plus 2 nM	ND[a]	195 (15)
20 nM	229 (0)	158 (31)
200 nM	199 (13)	127 (45)
2 μM	180 (21)	119 (48)

[a] ND, not determined.

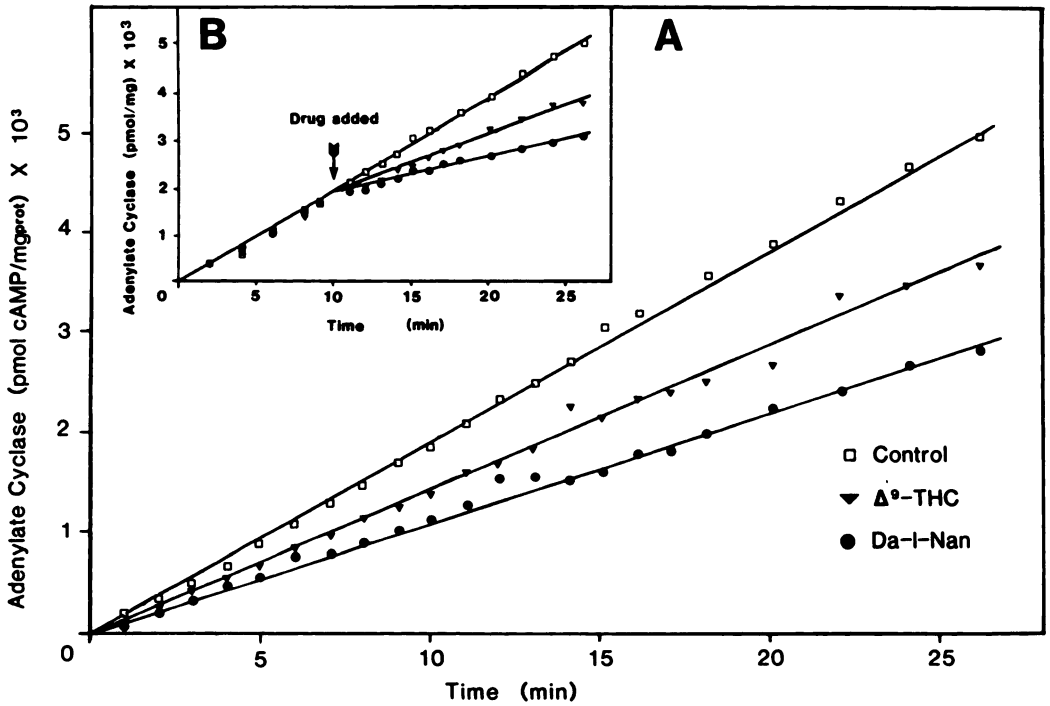

FIG. 1. *Kinetics of adenylate cyclase in the presence of cannabimimetic agents*

A, adenylate cyclase reaction mixtures (1750 μl) containing 100 nM secretin plus either vehicle, 5 μM Δ⁹-THC or 5 μM Da-*l*-Nan, were warmed to 30°. Membranes in HME buffer (350 μg of protein in 750 μl) were warmed for 2 min at 30° and then added to the reaction mixtures at time = 0 min. Aliquots of 100 μl were removed to "stop solution" at the indicated times. B, reaction mixtures and protein were prepared and the reaction proceeded as in A, except that cannabinoid drugs and vehicle were omitted. At time = 10 min, 75 μl of drugs were added, and the reaction continued. Aliquots were removed at the indicated times. These experiments are representative of four such experiments using similar protocols.

TABLE 2

Reversibility of cannabinoid inhibition of adenylate cyclase

Membranes (800 μg of protein) were treated at 30° in a 400-μl mixture consisting of 50 mM Na HEPES, pH 8.0, 1 mM EDTA, 5 mM $MgCl_2$, 0.1 mg/ml fatty acid-deficient bovine serum albumin, 50 μM ATP, 100 μM GTP, and either vehicle, 1 μM Δ⁹-THC or 100 nM Da-*l*-Nan. After 5 min, the mixtures were diluted 10-fold with 1 mg/ml fatty acid-deficient bovine serum albumin in HME buffer and sedimented at 100,000 × *g* for 20 min. The membranes were resuspended in 800 μl of HME buffer, and 20-μl aliquots were assayed for adenylate cyclase activity in the presence of the indicated additives. Numbers in parentheses are the per cent inhibition compared with the activity for secretin alone within each experimental group.

Treatment	Adenylate cyclase		
	Vehicle	Δ⁹-THC	Da-*l*-Nan
	pmol/min/mg		
Assay addition			
Secretin (500 nM)	172	165	164
Plus Δ⁹-THC			
300 nM	159 (8)	147 (11)	144 (12)
1 μM	147 (15)	136 (18)	142 (13)
3 μM	141 (18)	134 (19)	138 (16)
Plus Da-*l*-Nan			
10 nM	127 (26)	131 (21)	104 (37)
100 nM	99 (43)	89 (46)	76 (54)
1 μM	89 (48)	85 (48)	68 (58)
Plus carbachol			
300 μM	137 (20)	137 (17)	114 (30)

TABLE 3

Lack of cannabimimetic drug effects on substrate affinity

	V_{max}	K_m (Mg-ATP)	K_m (Mg-ATP) at 0.5 mM Mg^{2+}
	pmol/min/mg	*μM*	*μM*
Secretin (500 nM)	189 ± 43[a]	72 ± 4.5	70 ± 15.2
+ Da-*l*-Nan (1 μM)	136 ± 31[b]	58 ± 8.3[c]	77 ± 1.9[c]
+Δ⁹-THC (1 μM)	81 ± 11[d]	69 ± 3.0[c]	64 ± 15.0[c]

[a] Values are means ± standard error for $n = 3$.
[b] Significantly different at $p < 0.05$.
[c] Not significantly different at $p < 0.05$.
[d] Significantly different at $p < 0.01$.

tion (Fig. 1A), or were added after the hormone-stimulated reaction had progressed for 10 min (Fig. 1B). The inhibition was readily reversed upon removal of the compounds. In the experiment shown in Table 2, membranes were treated with maximally effective concentrations of cannabimimetic compounds under adenylate cyclase assay conditions. Dilution and sedimentation were used to separate membranes from unbound drugs. Subsequent assay of the membranes demonstrated that hormone-stimulated activity was not reduced in the membranes that had been treated with cannabimimetic agents compared to the control. Furthermore, concentration-dependent inhibition was produced, consistent with that exhibited in control membranes. Inhibition by submaximal concentrations of cannabimimetic compounds was also submaximal in those membranes that had been

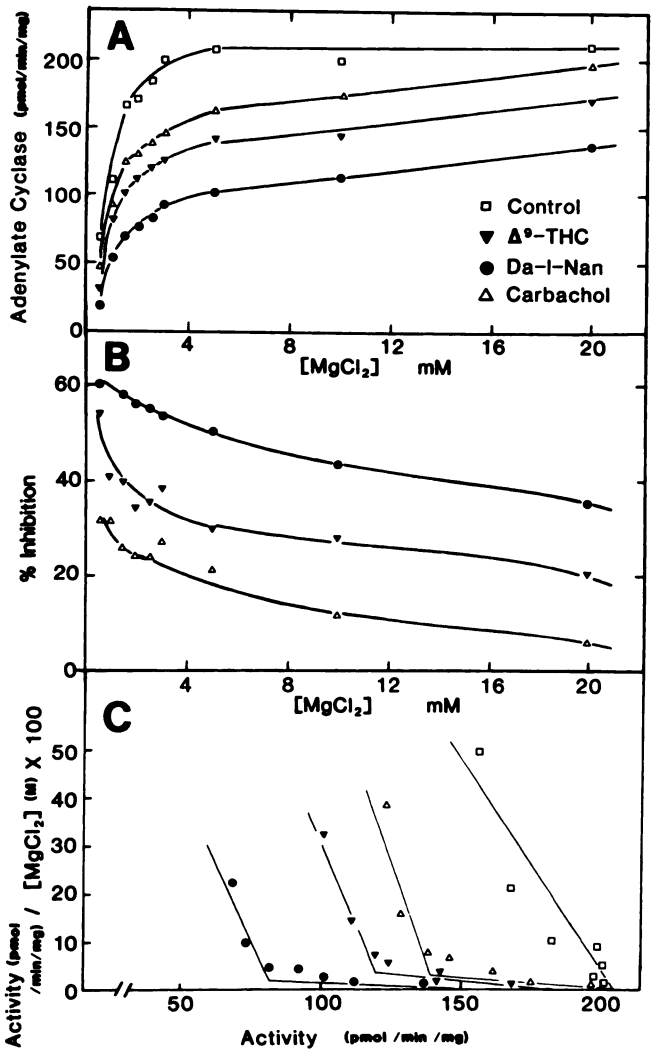

FIG. 2. *Magnesium ion requirements in the presence of inhibitory agents*

A, adenylate cyclase activity was measured as described in the text except that secretin was present at 500 nM, EDTA was present at 100 μM, and $MgCl_2$ was added at 0.5 to 20 mM. The data are plotted as the concentration of $MgCl_2$ added. This experiment is representative of three such experiments. B, the data in A were replotted to indicate the per cent inhibition of activity with respect to control. C, Eadie-Hofstee plots were constructed from these data. The concentrations of free Mg^{2+} were calculated using stability constants of 7.3×10^4 M^{-1} for Mg-ATP and Mg-GTP, and 2.5×10^6 M^{-1} for Mg-EDTA (20). The binding of Mg^{2+} to Na HEPES was assumed to be negligible.

previously treated with these inhibitory agents. Thus, cannabimimetic inhibition of adenylate cyclase is not the result of irreversible alterations in the enzyme protein or annular lipid structure. It is difficult to assess how rapid the reversal of the cannabimimetic inhibition actually is, because no known cannabinoid antagonists are available. However, sedimentation experiments have established that reversal is within minutes and does not require several washes of the membranes.

The cannabimimetic inhibition of adenylate cyclase was observed for both basal and hormone-stimulated adenylate cyclase activities (1). In addition, inhibition by these agents also occurred in the presence of forskolin, a compound believed to activate adenylate cyclase by an action on the catalytic protein (2). It was therefore of interest to determine if cannabimimetic agents affected the K_m for substrate. Adenylate cyclase uses Mg-ATP as substrate in its normally regulated state. In addition to being required as a chelate of ATP, Mg^{2+} probably acts at one or more sites in the enzyme complex as a regulator of activity (17–19). Therefore, the kinetic constants were determined by varying the concentration of Mg-ATP at several fixed concentrations of free Mg^{2+}. Double reciprocal plots constructed from such data were linear and intersected the abscissa to the left of the ordinate. Secondary replots of the slopes and intercepts were also linear, such that the data could be extrapolated to infinite Mg^{2+}. These data, appearing in Table 3, demonstrate that the K_m for Mg-ATP was not significantly altered by the presence of either Δ^9-THC or Da-*l*-Nan. As can be seen in Fig. 2, low concentrations of Mg^{2+} were optimal for inhibition of the enzyme by cannabimimetic or muscarinic agents. These agents also did not alter the K_m for Mg-ATP when $MgCl_2$ was added to the assay at 0.5 mM.

Regulation by divalent cations. The effects of increasing Mg^{2+} concentration in the presence and absence of cannabimimetic and muscarinic agents are shown in Fig. 2. The ability of these agents to inhibit secretin-stimulated adenylate cyclase activity was reduced as the Mg^{2+} concentration was increased. The Eadie-Hofstee plot determined for free Mg^{2+} in the presence of secretin alone was linear with a K_d for free Mg^{2+} at 100 μM (Fig. 2C). However, in the presence of muscarinic or cannabimimetic drugs, the plots were biphasic. The half-maximal concentrations of free Mg^{2+} averaged 75 μM and 1.8 mM Mg^{2+}. These data suggest that, in the presence of a stimulatory hormone, one site for divalent cations predominantly regulates adenylate cyclase activity. The inhibitory hormones may either unmask a negatively cooperative regulation or allow regulation by divalent cations at an additional site. These experiments indicate that the cannabimimetic agents act in the same manner as inhibitory hormones to modify the effects of divalent cations on adenylate cyclase activity.

The substitution of Mn^{2+} as the divalent cation resulted in concentration curves such as shown in Fig. 3. It should be noted that the increase in adenylate cyclase activity follows the increase in the concentration of substrate as well as of free Mn^{2+}, to a maximum at 490 μM Mn-ATP and 415 μM Mn^{2+} (calculated for 1.2 mM added $MnCl_2$). [Stability constants used were: 5×10^{11} M^{-1} for Mn-EDTA and 10^5 M^{-1} for Mn-ATP and Mn-GTP (20).] In this figure, the greatest inhibition by Da-*l*-Nan occurred at the lowest concentrations of divalent cation. The calculated free Mn^{2+} concentration does not exceed 20 μM until greater than 500 μM total $MnCl_2$ is added, at which concentration, the inhibition is reduced nearly by half. Increasing the $MnCl_2$ concentration to greater than 2.0 mM abolished the inhibitory action of both cannabimimetic and muscarinic agents; however, the hormone-stimulated activity was also greatly reduced at those concentrations (data not shown). A similar loss of the inhibitory response with increasing Mn^{2+} concentration also occurred in the presence of 0.5 mM $MgCl_2$

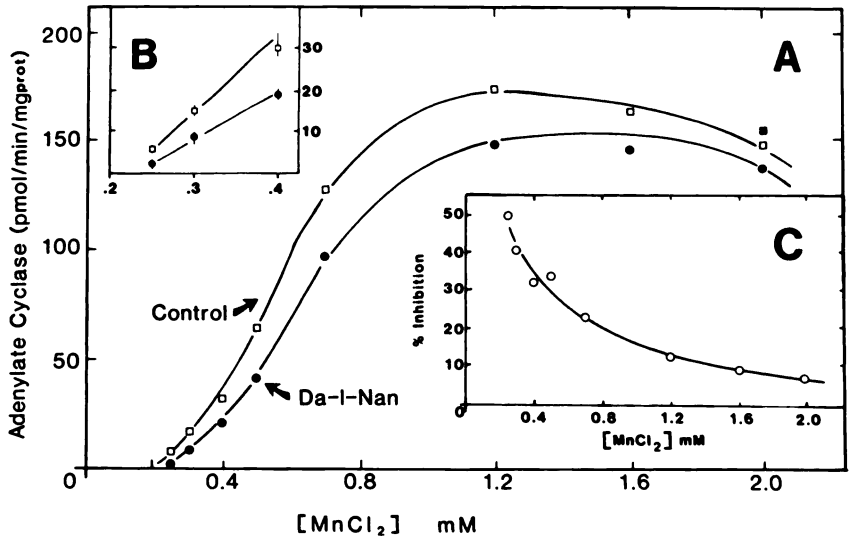

FIG. 3. *Manganese ion requirements in the presence of cannabimimetic agents*

A, the adenylate cyclase assay was modified by omitting $MgCl_2$, reducing the EDTA to 200 μM, and adding 1 mM dithiothreitol and the indicated concentrations of $MnCl_2$. Secretin was present at 300 nM. The secretin-stimulated activity in the presence of 4 mM $MgCl_2$ is indicated by the filled square. This representative experiment was reproduced twice as described. B, the scale is expanded to show the first three data points. The bars represent the range of triplicate samples. C, the data from A were replotted as the per cent inhibition with respect to control.

(data not shown). It appears that a site for regulation of hormone-stimulated adenylate cyclase is able to accommodate Mn^{2+} as the divalent cation. The inhibition produced by cannabimimetic agents, as shown here, is sensitive to concentrations of Mn^{2+} that are below those required for maximal stimulation.

Regulation by guanine nucleotides. It has been observed in other systems that hormonal inhibition of adenylate cyclase is optimal at concentrations of GTP exceeding those necessary for stimulation of activity (7, 8). An increase in basal and secretin-stimulated adenylate cyclase activity was observed upon increasing GTP concentrations above 10 nM (Fig. 4). Perhaps contaminating or membrane-bound guanine nucleotides can account for the enzyme activity observed in the absence of exogenous GTP. For both basal and secretin-stimulated enzymes, half-maximal activity occurred at about 50 nM GTP. In the experiments shown, a decrement in activity appeared at 10–100 μM GTP. This response is not consistently observed, and when it occurs, the decline rarely exceeds 15% of the activity at 1 μM GTP. The guanine nucleotide concentration requirement was tested for inhibition by Δ^9-THC and Da-*l*-Nan and compared to that for carbachol. Inhibition by cannabimimetic and muscarinic agents was observable at 100 nM GTP and half-maximal at 800 nM GTP. It is apparent that the requirement for guanine nucleotide for inhibition is identical for both types of agents. This suggests that the GTP-binding site associated with inhibition by muscarinic hormones is also involved in inhibition by cannabimimetic drugs.

Substitution of the nonhydrolyzable analog Gpp(NH)p for GTP failed to support the inhibition by either carbachol or the cannabimimetic agents (Table 4). This was true over a range of guanine nucleotide concentrations and for several $MgCl_2$ concentrations that were optimal for activation of adenylate cyclase. Enzyme that had been stably activated by Gpp(NH)p and then washed free of the guanine nucleotide also failed to respond to either cannabimimetic or muscarinic inhibitors (Fig. 5). In this experiment, Gpp(NH)p treatment resulted in enzymatic activity 3-fold greater than that of GTP-treated membranes. No inhibition was observed whether these membranes were assayed in the absence of nucleotide, or in the presence of nucleotide or nucleotide plus secretin.

Recent investigators using forskolin as an activator of adenylate cyclase have demonstrated that, under certain assay conditions, an inhibition of adenylate cyclase activity can be observed when Gpp(NH)p is used as the guanine nucleotide (9, 21–23). We recently demonstrated that cannabimimetic agents inhibit adenylate cyclase activity in the presence of forskolin plus GTP (2). It was of interest to see if, in this system, assay conditions could be modified such that inhibition could be observed in the presence of forskolin and Gpp(NH)p. Fig. 6 demonstrates that a short incubation with less than 100 μM free Mg^{2+} and high concentrations of forskolin resulted in an enzyme that does not exhibit activation by Gpp(NH)p. Under these conditions, carbachol and Da-*l*-Nan produced as much as 18 and 34% inhibition, respectively, compared with controls. Numerous experiments, conducted at higher $MgCl_2$ or lower forskolin concentrations or longer assay times, resulted in an enzyme that was activated by Gpp(NH)p. In those assays, no inhibition could be observed upon addition of either carbachol or cannabimimetic agents.

DISCUSSION

The present investigation has examined the mechanism by which cannabimimetic agents interact with adenylate cyclase. The approach has been to examine the effects that these drugs have on the regulation of this

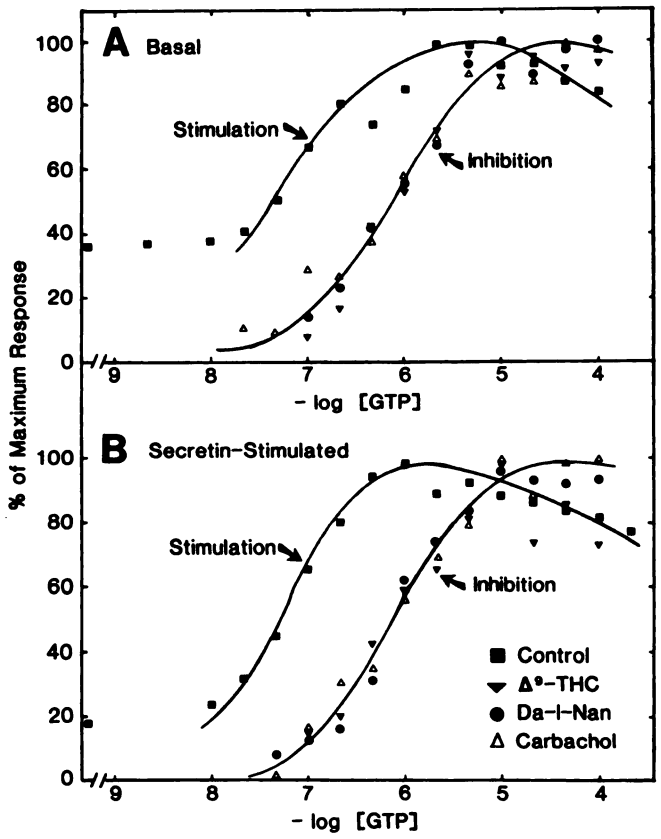

FIG. 4. *GTP requirement for stimulation and inhibition of adenylate cyclase*

A, basal activity was determined as described in Experimental Procedures except that ATP was at 250 μM and [α-^{32}P]ATP was at 1.5 μCi/tube. GTP was added at the indicated concentrations. Each point represents the average of two experiments in which triplicate determinations were made. Maximum stimulation (100%) was 31.4 and 33.3 pmol/min/mg. Maximum inhibition (100%) was 35 and 31% for 3 μM Δ⁹-THC, 54 and 56% for 3 μM Da-*l*-Nan, and 41 and 33% for 300 μM carbachol. B, secretin (500 nM) was present in the adenylate cyclase reaction mixtures and GTP was added at the indicated concentrations. Each point represents the average of three experiments (duplicate determinations each) using two different membrane preparations. Maximum stimulation ranged between 158 and 266 pmol/min/mg. Maximum inhibitions ranged between 24 and 32% for 3 μM Δ⁹-THC, 46 and 56% for 3 μM Da-*l*-Nan, and 27 and 28% for 300 μM carbachol.

enzyme by hormones, guanine nucleotides, and divalent cations. Inhibition of adenylate cyclase by hormones functions as a regulatory mechanism in a variety of cell types (for review and references, see Refs. 7 and 8). A description of the components of this enzyme complex and their regulatory functions can be found in current reviews (8, 9).

We have reported here that cannabimimetic inhibition of adenylate cyclase is observed immediately upon addition of the agent and is readily reversible upon its removal. The data presented here also demonstrate that the inhibition is not due to modification of the catalytic protein's affinity for substrate. Further investigation has analyzed the behavior of cannabimimetic compounds for characteristics consistent with a mechanism of receptor-mediated inhibition of adenylate cyclase. As an internal

TABLE 4

Inability of inhibitory agents to alter adenylate cyclase activity in the presence of Gpp(NH)p and high Mg^{2+}

Adenylate cyclase was assayed using conditions described in the text, except that GTP was absent and $MgCl_2$ was 10 mM. Gpp(NH)p was included at the indicated concentrations, carbachol was present at 300 μM, and the cannabimimetic drugs were present at 3 μM. This experiment is representative of five similar experiments in which the concentrations of Gpp(NH)p ranged from 10 nM to 1 mM and $MgCl_2$ was either 5, 10, or 20 mM.

Gpp(NH)p	Adenylate cyclase			
	Control	Δ⁹-THC	Da-*l*-Nan	Carbachol
	pmol/min/mg			
0	12	12	11	11
100 nM	93	96	85	87
500 nM	113	118	123	114
5 μM	137	140	144	133
50 μM	146	142	147	138

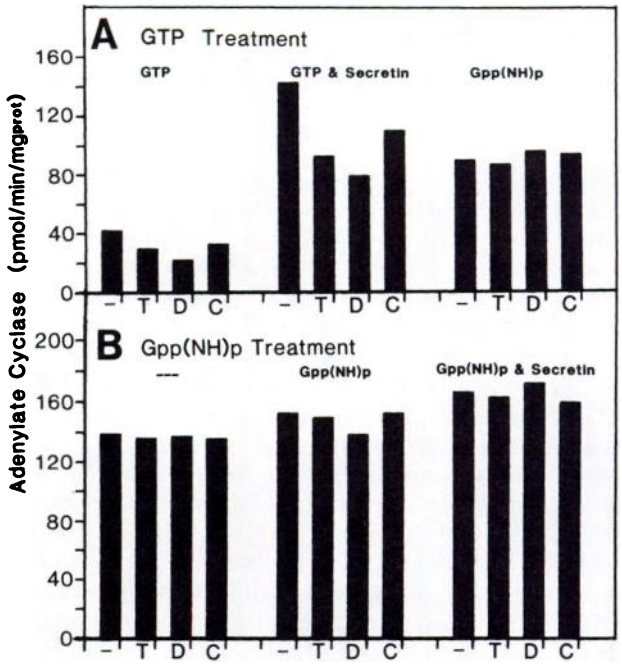

FIG. 5. *Failure to inhibit adenylate cyclase activity in enzyme previously activated by Gpp(NH)p*

Membranes were incubated at 23° for 20 min in a 500-μl volume containing 20 mM Na HEPES, pH 8.0, 1 mM EDTA, 20 mM $MgCl_2$, 0.5 mM ATP, and either 100 μM GTP (A) or 100 μM Gpp(NH)p (B). The mixture was diluted 6-fold with 20 mM Na HEPES and sedimented at 100,000 × *g* for 20 min. The centrifuge tube was wiped dry and the membrane pellet was resuspended in 1 ml of HME buffer. Aliquots of 20 μl were assayed in the presence of 100 μM GTP or Gpp(NH)p, 500 nM secretin, and 3 μM Δ⁹-THC (*T*) or Da-*l*-Nan (*D*), and 300 μM carbachol (*C*) or vehicle (—) as indicated. This experiment was repeated with identical results.

control, the muscarinic cholinergic inhibition of adenylate cyclase has been used throughout the study.

Both muscarinic and cannabimimetic compounds exhibited their greatest inhibitory ability at low divalent cation concentrations. It has been observed that stimulatory hormones increase the apparent affinity for Mg^{2+} for activation of adenylate cyclase (17, 19). This is also

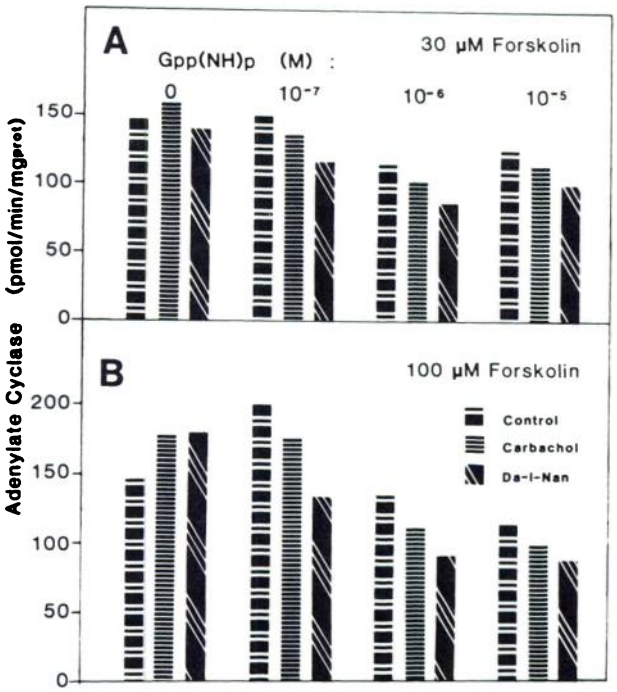

FIG. 6. *Inhibition of adenylate cyclase activity in the presence of forskolin and Gpp(NH)p*

The assay conditions were modified by reducing the ATP to 250 μM and the $MgCl_2$ to 1.35 mM, and conducting the reaction for 5 min at 30°. Forskolin was present at 30 μM (A) or 100 μM (B) and Gpp(NH)p was either absent or at 0.1, 1, or 10 μM as indicated. Carbachol was included at 300 μM and Da-*l*-Nan was at 3 μM as indicated. This experiment is representative of three similar experiments.

true for the stimulation of neuroblastoma adenylate cyclase by prostanoid stimulators and by secretin (24). The addition of either muscarinic or cannabimimetic compounds to the hormone-stimulated enzyme resulted in the appearance of a second site having lower apparent affinity for Mg^{2+}. This complex regulation by divalent cations was reported also for inhibition of pituitary adenylate cyclase by dopamine and for inhibition by hormones in other systems (25). At least two divalent cation sites, one of which may be on the catalytic subunit, have been suggested from data determined using S49 lymphoma wild-type and variant cells (18, 19). Perhaps the action of inhibitory hormones unmasks the cation regulatory function of a site on G_s or G_i that had been rendered cryptic by the action of stimulatory hormones.

In the present study, the inhibition by cannabimimetic agents was particularly sensitive to antagonism by Mn^{2+}. This was previously observed for platelet adenylate cyclase, in which 0.4 to 2.0 mM $MnCl_2$ augmented the prostaglandin stimulation but impaired the α-adrenergic inhibition of the enzyme (26). A similar phenomenon was reported for hamster adipocyte enzyme (27). These findings could be interpreted as an indication that there are two binding sites associated with G_s and G_i, having different affinities for divalent cation. Alternatively, the presence of a Mn^{2+} at a single site may influence the ability of the system to be under predominantly stimulatory or inhibitory influence. Whatever the precise mechanism, the action of cannabimimetic compounds mimics that of inhibitory hormones as observed for the divalent cation regulation of adenylate cyclase.

Both G_s and G_i are GTP-driven coupling complexes. The concentrations of GTP for inhibition by hormones has been reported for numerous systems to be greater than that for stimulation by hormones (7, 8). This has been shown here for carbachol and for the cannabimimetic agents. Sites on both G_s and G_i bind Gpp(NH)p, and stable dissociation of both complexes proceeds in the presence of divalent cations (9). However, lower Mg^{2+} concentrations were required for dissociation of G_i (23). In addition, an allosteric site other than that required for dissociation of the G_s complex has been suggested as a requirement for activation of the catalytic component (17). Thus, the competition for interaction of the GTP-binding proteins with either the catalytic protein or the other proteins of the G_s and G_i complexes may depend on the divalent cation concentration. The use of forskolin as a tool to augment catalytic activity has allowed the observation of the inhibitory actions of guanine nucleotide analogs (21–23). In the present investigation, it was demonstrated that, at concentrations of Mg^{2+} that promoted Gpp(NH)p activation of the enzyme, both carbachol and the cannabimimetic agents failed to inhibit adenylate cyclase. In contrast, when the Mg^{2+} was reduced and the period of incubation was limited, the effects of Gpp(NH)p were probably restricted to the G_i subunit, and inhibition was produced by either class of inhibitors. These results are consistent with those reported by others (21–23).

It can be inferred from these results that the action of cannabimimetic agents must be via the regulatory mechanisms that are operative in the receptor-mediated inhibition of adenylate cyclase. One possibility is that cannabimimetic compounds interact with an inhibitory hormone receptor protein that regulates the function of G_i. Further evidence supporting this view comes from our recent finding that the islet-activating protein of *Bordetella pertussis* toxin can ameliorate the cannabinoid inhibition of adenylate cyclase.[2]

Actions of cannabinoid drugs other than the inhibition of adenylate cyclase may be due to an interaction with plasma membrane lipids. It is possible that cannabinoid compounds may disrupt or promote formation of lipid domain structures. Alternatively, cannabinoid compounds may interact specifically with a particular lipid species required in the annular lipid structure of certain proteins. Further study is necessary to obtain evidence for or against these possibilities as additional influences of cannabinoid drugs on neuronal functions.

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Send reprint requests to: Allyn C. Howlett, Ph.D., Department of Pharmacology, St. Louis University School of Medicine, 1402 S. Grand Blvd., St. Louis, MO 63104.