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REGULATION OF ADENYLATE CYCLASE BY CANNABINOID DRUGS

INSIGHTS BASED ON THERMODYNAMIC STUDIES

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Abstract—The abilities of lipophilic cannabinoid drugs to regulate adenylate cyclase activity in neuroblastoma cell membranes were analyzed by thermodynamic studies. Arrhenius plots of hormone-stimulated adenylate cyclase activity exhibited a break point at 20°. The break point was reduced to 14° by benzyl alcohol, consistent with results from other laboratories that have correlated this response with the increase in membrane fluidity induced by benzyl alcohol. Because cannabinoid drugs partition into membrane lipids and alter membrane fluidity parameters in a number of model systems, it was of interest to examine the influence of Δ^9-tetrahydrocannibinol and cannabidiol on enzyme activity analyzed by the Arrhenius plot. Δ^9-Tetrahydrocannabinol, known to inhibit adenylate cyclase, failed to decrease the transition temperature either at 1 μM or at concentrations exceeding its aqueous solubility (30 μM), suggesting that Δ^9-tetrahydrocannabinol could not mimic the effects observed with benzyl alcohol. In contrast, 30 μM cannabidiol, which stimulated enzyme activity slightly, decreased the Arrhenius plot break point to 17.5°. The decrease in the transition temperature in response to benzyl alcohol or cannabidiol was not accompanied by a change in activation energies above or below the transition temperature. Δ^9-Tetrahydrocannabinol inhibits adenylate cyclase activity via G_i as does the muscarinic agonist carbachol (Howlett *et al.*, *Mol Pharmacol* **29**: 307–313, 1986). Both carbachol and Δ^9-tetrahydrocannabinol decreased the enthalpy and entropy of activation. The net free energy of activation at 37° was increased in the presence of both of these inhibitory agonists. These data suggest that, for the entropy-driven hormone-stimulated adenylate cyclase enzyme, less disorder of the system occurs in the presence of regulators that inhibit the enzyme via G_i. In summary, thermodynamic data suggest that cannabidiol can influence adenylate cyclase by increasing membrane fluidity, but that the inhibition of adenylate cyclase by Δ^9-tetrahydrocannabinol is not related to membrane fluidization.

Adenylate cyclase is a membrane-associated enzyme complex comprising a catalytic protein, the stimulatory and inhibitory G-proteins G_s and G_i, and receptors that activate these G-proteins (for review see Refs 1 and 2). The catalytic protein is a glycoprotein having a large hydrophobic surface area and is thus believed to span the membrane. The G-proteins are heterotrimers whose α subunits dissociate from the β and γ subunits upon binding of GTP. The interaction of the α subunits with the membrane varies within the family of G-proteins. The α subunits of G_s and G_i are relatively firmly associated with plasma membranes, although solubilized α_i can behave as a monomer in the absence of detergent [3]. The β and γ proteins may be more strongly associated with the plasma membrane, since they associate readily with phospholipid vesicles and aggregate in the absence of detergent [3]. The secondary structures of β-adrenergic and muscarinic receptors that regulate G_s and G_i, respectively, have been deduced recently from their primary amino acid sequences [4, 5]. These receptors are glycoproteins believed to have seven transmembrane-spanning regions that form a pore-like structure to accommodate the hormone.

The protein components of the adenylate cyclase system are envisioned to diffuse within the two-dimensional structure of the membrane and to interact transiently in a "collision coupling" model [2] of enzyme activation. Upon binding of the hormone, the receptor–hormone complex interacts with the G-protein to facilitate binding of GTP to the α subunit and its consequent dissociation from the β and γ subunits (see Refs 1 and 2). A single free α_s may potentially activate a number of catalytic proteins before it is inactivated by the hydrolysis of GTP to GDP. Continuation of the cycle depends upon reformation of the GDP-bound G-protein heterotrimer and its interaction with a receptor–hormone complex. A parallel process occurs when an inhibitory receptor–hormone complex interacts with G_i. It is unclear whether inhibition results from a direct interaction of α_i with the catalytic protein. Evidence exists for a mechanism of inhibition by which the concentration of free α_s subunits is reduced as a consequence of equilibrium binding with the β and γ subunits released from G_i [1].

It can be envisioned that the membrane lipid environment may be critical for successful protein–protein interactions within the adenylate cyclase complex. Many examples appear in the literature of lipophilic and amphipathic compounds that alter adenylate cyclase activity as a function of their interaction with the plasma membrane (see Refs 6 and 7 for review). Some of these agents have been demonstrated by fluorescence polarization or ESR probe

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analysis to produce alterations in membrane fluidity. In parallel studies, the influence of membrane fluidity on adenylate cyclase activity can be characterized by analyzing the Arrhenius plots of enzyme activity as a function of temperature [6]. Membrane-perturbing agents need not penetrate the entire lipid structure of the cell to cause an effect. Enzymes tend to be clustered in fluid lipid domains, the modification of which could affect enzyme activity. Integral membrane proteins also tend to be surrounded by strongly associated annular lipids, some of which may have quite specific structural specificity for optimal activity. Examples are found in the literature that describe a selectivity for particular phospholipids or neutral lipids that permit hormone-regulated adenylate cyclase activity [8–11].

The objective of the present study was to examine the mechanism(s) by which lipophilic cannabinoid drugs influence adenylate cyclase activity. Δ^9-THC* is the primary psychoactive compound isolated from extracts of marihuana (*Cannabis sativa*) [12]. Other cannabinoid compounds, notably CBD, do not produce a psychological "high" at physiological concentrations, but have been shown to be anticonvulsant and to produce CNS depression at high doses [12, 13]. The mechanisms of action for these effects of cannabinoid compounds are not clear [14]. My laboratory has demonstrated that psychoactive cannabinoid drugs inhibit adenylate cyclase activity in membranes from cultured neuronal cells [15, 16]. We have further demonstrated that this effect requires G_i [17] and a membrane-associated cannabinoid receptor [18, 19]. In contrast, CBD and many other cannabinoid drugs lacking psychoactivity are unable to inhibit adenylate cyclase [15, 20]. It has been proposed that the lipid bilayer of the neuronal membrane may be a primary site of action of the cannabinoid compounds based on the data that these compounds have a high membrane: buffer partition coefficient [12, 14]. Cannabinoid drugs have been demonstrated to alter membrane fluidity parameters in synthetic lipid and synaptosomal lipid vesicles [21–24]. If cannabinoid drugs were to alter membrane properties within the lipid domain of the enzyme or to interact specifically with the strongly associated annular lipids, then adenylate cyclase activity would be modified. The present investigation examined the effects of Δ^9-THC and CBD on adenylate cyclase in membranes from neuronal cells by analysis of Arrhenius plots of activity. Thermodynamic parameters calculated from these data can provide insights into both the influence of membrane fluidity on hormone-stimulated adenylate cyclase and the mechanism by which inhibitory hormones regulate adenylate cyclase.

EXPERIMENTAL PROCEDURES

Cell culture and membrane preparation. The N18TG2 (passages 27 through 50) and N4TG1 (passages 30 through 40) clones of the C1300 neuroblastoma were used for these experiments. Cells were grown in Ham's F12:Dulbecco's modified Eagle's medium (1:1) containing 10% donor calf bovine serum that had been heat-inactivated (Flow Laboratories Lot No. 29 141102 or Hazleton Research Products Lot No. 12 143010). The medium also contained either penicillin (50 units/ml) plus streptomycin (50 μg/ml), or gentamycin (20 μg/ml). Cells were harvested, and a plasma membrane fraction was prepared by differential and sucrose density gradient sedimentations [16]. The membranes were aliquoted for storage at −80°, and thawed immediately prior to use. Over the course of these experiments, a single N4TG1 and three separate N18TG2 plasma membrane preparations were used. The results for N18TG2 membranes did not vary between the three preparations.

Adenylate cyclase determinations at multiple temperatures. Experiments were performed using a variable temperature block originally described by Gordon and Mobley [25] and fabricated in our laboratory. The main body of the block is a solid piece of aluminum with dimensions of 70 × 7 × 4.5 cm. Wells 3.5 cm deep and 1 cm in diameter were drilled in triplicate at evenly spaced intervals (4 mm). Borosilicate glass test tubes fit loosely in the well providing room for a temperature-equilibrated water reservoir. At each end of the block, perpendicular aluminum extensions with dimensions of 4.5 ×7 × 7.5 cm project downward. All exposed block faces were covered with styrofoam. To establish a temperature gradient, one extension was placed in a Temp-Blok module heating block and the other in a circulating ice water bath. After equilibration was established in 2.5 hr, temperatures were measured in test tubes containing 1 ml of H_2O in one row of the triplicate wells using a thermometer incremented in 0.2° intervals.

Adenylate cyclase activity was determined in a final volume of 120 μl containing 50 mM sodium HEPES, pH 8.0, 100 μM 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone, 1 mM EDTA, 5 mM $MgCl_2$, 3 mM potassium phosphoenolpyruvate, 100 μg/ml bovine serum albumin, 10 μg/ml pyruvate kinase, 500 μM ATP, 100 μM cAMP, 100 μM GTP, 650 nM secretin and 1.0 μCi of [^{32}P]ATP. Additional compounds were present as described. Test tubes containing the reaction mixture (100 μl) were allowed to equilibrate in the variable temperature block for 13.6 min. Adenylate cyclase activity was then initiated by the addition of membranes (exactly 15 μg protein) in 20 μl. The membrane protein concentration was identical for all experiments such that a constant ratio of drug to membrane proteins and lipids could be maintained for each experiment. The reaction was terminated after 20 min, and [^{32}P]cAMP was isolated from sequential Dowex 50W and alumina columns as previously described [16]. Duplicate determinations were made for each temperature point, and the data were averaged prior to analysis.

These studies were performed at temperatures within a range known to have linear hormone-stimulated activity for the 20-min assay time. Initial studies demonstrated that, in the presence of GTP plus the stimulating hormone secretin, enzymatic activity was linear for at least 25 min at temperatures ranging from 12° to 37° (data not shown). No lag was

* Abbreviations: THC, tetrahydrocannabinol; CBD, cannabidiol; E_a, activation energy; and HEPES, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid.

observed at lower temperatures in the presence of the stimulatory hormone. At 45°, the activity was linear for only 10 min before declining, probably as a result of thermal denaturation of the proteins. Thus, the maximum temperature employed was 37°.

Statistical analysis of the data. Experiments were analyzed in the form of an Arrhenius plot. The break point (or points) on the curve was determined using a statistical method developed by Jones and Molitoris [26]. This procedure fits two lines that join at a break point that can vary continuously within the range of the data. The method iteratively solves for the value of the break point that minimizes the residual sum of squares for an equation derived from the equations for the two straight lines on either side of the break point. The assumption that the error in the data points is independently distributed about the joined lines with a Gaussian distribution with constant variance has been demonstrated for the data in this study (i.e. all normalized residuals were less than 1 in absolute value). An approximate F test was calculated to determine whether the broken line was a significantly better fit than a single straight line. The F values for the individual experiments ranged from 11 to 193 with a median value of 74, indicating that under no experimental conditions was a single straight line preferable to one with a break point. Separate analyses above and below the major break point were used to determine the existence and location of a second break point. These determinations were facilitated by the use of a BASIC computer program for the IBM-PC obtained from Dr R. H. Jones, University of Colorado Health Sciences Center, which was rewritten in our laboratory to include graphics for the Apple II Plus computer.

For each individual experiment, E_a was calculated using the formula: $E_a = -2.3R\ (m)$ where m is the slope of the Arrhenius plot and R is the gas constant [27]. The enthalpy change of activation, $\Delta H^{\dagger}$, was calculated above and below the transition temperature using the formula: $\Delta H^{\dagger} = E_a - RT$, where T is 303°K and 285°K respectively. The free energy change of activation, $\Delta G^{\dagger}$, was calculated using the formula: $\Delta G^{\dagger} = -RT2.3 \log_{10} [A(h)/kT]$ where A is the activity of adenylate cyclase in molecules/sec at either 303°K or 285°K, k is the Boltzmann constant and h is Planck's constant [27]. The entropy change of activation, $\Delta S^{\dagger}$, was calculated at 303°K or 285°K using the formula $\Delta S^{\dagger} = -(\Delta G^{\dagger} - \Delta H^{\dagger})(T^{-1})$. The means of data from experimental groups were compared with control using Student's t-test for unpaired observations having either equal or unequal variances as determined by an F test [28].

Materials. The National Institute on Drug Abuse supplied the Δ^9-THC (Lot No. 3498-183) and CBD (Lot No. 5389-196a) in absolute ethanol, and these were used within 24 months after receipt. These compounds were stored at 10 mg/ml in ethanol at −20° until use. Aliquots were taken to dryness under a stream of N_2 in Regisil-treated glass test tubes and vigorously resuspended to 100 μM in 50 mg/ml fatty acid-deficient bovine serum albumin warmed to 37° as previously described [16]. This suspension or control bovine serum albumin was diluted directly into the reaction mixture in Regisil-treated glass test tubes. Benzyl alcohol was Sigma B-2263 (Lot No. 44F-3514).

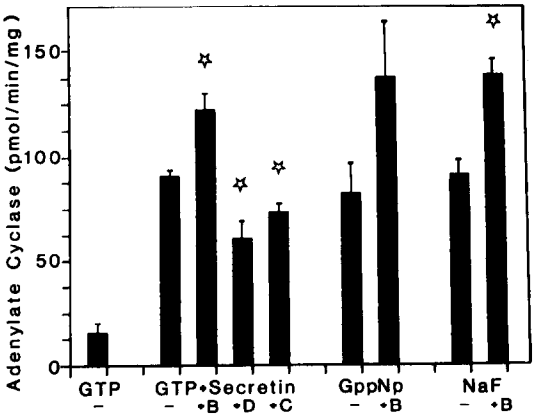

Fig. 1. Effects of benzyl alcohol on adenylate cyclase activity in neuroblastoma plasma membranes. Adenylate cyclase activity was determined at 30° as described in the text. The drug concentrations were: 100 μM GTP, 3 μM secretin, 30 mM benzyl alcohol (B), 1 μM desacetyllevonantradol (D) and 300 μM carbachol (C). Values are means ± SE, N = 3. Stars indicate that the difference from data in the absence of the indicated additive is significant at $P < 0.05$. This experiment is representative of three experiments having qualitatively similar results.

RESULTS

To test for effects of alterations in membrane fluidity on adenylate cyclase activity in neuroblastoma membranes, benzyl alcohol was used as a membrane perturbant. Benzyl alcohol is a neutral organic alcohol which is known to partition into lipid bilayers and thereby increase bilayer fluidity and decrease the temperature at which lipid phase transitions occur [29, 30]. For this reason, benzyl alcohol has been used to assess the effects of increased membrane fluidity on the activity of adenylate cyclase and other integral membrane enzymes [6, 7, 30, 31]. Figure 1 shows that benzyl alcohol increased hormone-stimulated adenylate cyclase activity. Benzyl alcohol also significantly ($P < 0.05$) increased activity in the presence of NaF and showed a tendency to increase the response to the nonhydrolyzable GTP analog, guanylyl-5′-imidodiphosphate (GppNp). These agents act directly on the G_s protein to promote dissociation of the subunits such that α_s can activate the catalytic protein. The increase in enzymatic activity by benzyl alcohol was consistently observed, and ranged from 35 to 80% above control during the course of these studies. The optimal concentration of benzyl alcohol was 30 mM whether in the presence of hormones or G_s activators (data not shown). Benzyl alcohol concentrations exceeding 100 mM eliminated enzymatic activity altogether. This narrow concentration–response relationship is consistent with data reported for other systems [6, 7, 29, 30].

Also shown in Fig. 1 is the typical inhibition of adenylate cyclase activity that is observed upon addition of cannabinoid compounds such as

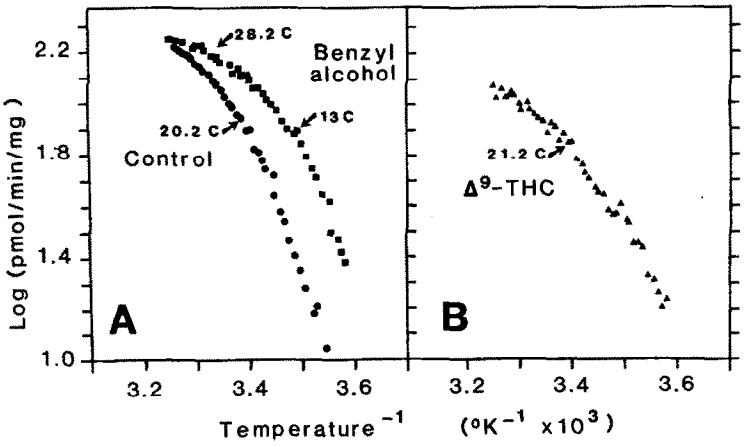

Fig. 2. Arrhenius plots of hormone-stimulated adenylate cyclase activity in neuroblastoma membranes. Individual experiments are shown in which adenylate cyclase activity was determined at intervals of temperature ranging between 5° and 35°; activity was analyzed as described in the text. (A) The specific activity of the control at 30° was 144 pmol/min/mg and the activation energies were 45 and 110 kJ/mol above and below the break temperature respectively. The specific activity at 30° in the presence of 30 mM benzyl alcohol was 170 pmol/min/mg and the activation energies were 49 and 105 kJ/mol. (B) The specific activity in the presence of 1 μM Δ^9-THC at 30° was 101 pmol/min/mg and the activation energies were 29 and 63 kJ/mol.

desacetyllevonantradol. The nantradol class of analogs of Δ^9-THC and related bicyclic and tricyclic cannabinoid analgetics have been demonstrated recently to act via a membrane-associated cannabinoid receptor [7, 32]. Shown for comparison is the inhibition of adenylate cyclase by carbachol, acting through the muscarinic receptor. Both of these receptors are believed to exert their effects by interacting with G_i to promote the dissociation of α_i from the β and γ proteins [5, 16, 17]. Benzyl alcohol either had no effect or slightly decreased the inhibition of adenylate cyclase by these agents (data not shown).

Hormone-stimulated adenylate cyclase activity was determined as a function of temperature and displayed in the form of Arrhenius plots. Figure 2A depicts a typical plot of secretin-stimulated activity, for which two straight lines could be described on either side of a single break at 20.2°. In the plot generated from an experiment in which benzyl alcohol was also included, the break in the Arrhenius plot was shifted to a lower temperature (13°), consistent with what has been observed by others [29–31]. This is interpreted to mean that a lipid phase change critical to adenylate cyclase activity is observed at a lower temperature when the bilayer structure is perturbed by benzyl alcohol. Enzyme activities were higher than for control at all temperatures, and the activation energies on either side of this transition temperature were similar to the control values (data not shown) as has been observed in other adenylate cyclase systems in which benzyl alcohol lowered the transition temperature by increasing membrane fluidity [29–31].

In contrast to the control plot, the data obtained in the presence of benzyl alcohol was fit better by a set of three straight lines (Fig. 2A). For each experiment containing benzyl alcohol, the adenylate cyclase activity either failed to increase or severely declined with temperature above 26 ± 2.6° (N = 3). Such behavior is generally indicative of thermal inactivation. Needham and colleagues [32] have shown previously that benzyl alcohol can promote the thermal inactivation of adenylate cyclase. These investigators suggested that the increased membrane fluidity destabilizes the tertiary structure of one of the enzyme subunits, probably the catalytic protein [32]. Alternatively, it has been proposed that benzyl alcohol displaces annular lipids at concentrations exceeding 60 mM since membrane fluidity changes that coincide with enzyme inhibition at high concentrations cannot be detected [29, 30].

The Arrhenius plot of adenylate cyclase activity in the presence of 1 μM Δ^9-THC is shown in Fig. 2B. The temperature at which the break occurred in the Arrhenius plot was 21.2°. The Arrhenius plots of enzyme activity in the presence of 30 μM Δ^9-THC were similar. This contrasts with what was observed when benzyl alcohol was added (Fig. 2A).

The results of multiple experiments were combined for statistical comparisons, as shown in Table 1. Benzyl alcohol decreased the transition temperature by greater than 5°. The activation energies above and below the transition temperature were not altered significantly by the presence of benzyl alcohol (data not shown). This is consistent with previous reports of decreased transition temperatures of hepatic membrane adenylate cyclase that occur using conditions shown by ESR to result in a fluidization of the membranes [6, 30]. CBD, which stimulates enzyme activity slightly [15], also decreased significantly the transition temperature (Table 1) without altering the activation energies (data not shown). This suggests that the behavior of CBD may resemble that of benzyl alcohol in its influence on the adenylate cyclase complex.

It is clear that Δ^9-THC at 1 μM, the concentration that is optimum for inhibition of adenylate cyclase, did not alter the break point of the Arrhenius plot

Table 1. Break point temperatures and activation energies for N18TG2 and N4TG1 adenylate cyclase

	Transition temperature (°C)	N
A. N18TG2		
Vehicle 1	19.8 ± 1.90	4
30 mM Benzyl alcohol	14.2 ± 1.33*	3
1 μM Δ^9-THC	20.9 ± 0.31	4
1 mM Carbachol	19.6 ± 0.94	5
Vehicle 2	21.2 ± 0.49	4
30 μM Δ^9-THC	21.7 ± 0.26	5
30 μM CBD	17.5 ± 0.23*	5
B. N4TG1		
Vehicle 1	19.8 ± 1.22	4
1 μM Δ^9-THC	18.5 ± 0.39	4
30 μM Δ^9-THC	18.5 ± 0.92	3

Values are means ± SE.
* Less than vehicle at the $P < 0.05$ level of significance.

when compared to the vehicle control. Δ^9-THC at 30 μM, a concentration that exceeds the aqueous solubility under these conditions [33], also did not reduce the transition temperature compared to the vehicle control. Carbachol, which inhibits adenylate cyclase via the muscarinic cholinergic receptor, was used as a negative control in these studies. This compound, which is not expected to accumulate in the membrane or to alter membrane fluidity, also failed to alter the transition temperature with respect to vehicle. These results demonstrate that inhibitory regulation of adenylate cyclase does not involve a change in the transition temperature that might result from a perturbation of membrane fluidity. The activation energies determined in the presence of these adenylate cyclase inhibitors were not significantly different from control above the transition temperature. However, the activation energy below the transition temperature was reduced significantly ($P < 0.05$) by carbachol [68 ± 1.2 kJ/mol vs control of 89 ± 6.6 kJ/mol]. A tendency to be decreased was evident for 30 μM Δ^9-THC [79 ± 9.0 kJ/mol vs control of 102 ± 9.6 kJ/mol].

Also examined was the N4TG1 neuroblastoma cell clone, which like N18TG2 is derived from the C1300 neuroblastoma, but which fails to respond to psychoactive cannabinoid drugs with an inhibition of adenylate cyclase [34]. It is believed that this cell line does not bear receptors for Δ^9-THC, and, therefore, any effects that cannabinoid drugs would have on adenylate cyclase activity would not be expected to be receptor mediated. The N4TG1 clone exhibited a poor response to secretin or VIP (data not shown), and, thus, prostaglandin E_1 was used as the hormonal stimulator. The break point of the Arrhenius plots of N4TG1 adenylate cyclase stimulated by prostaglandin E_1 was the same as that for the N18TG2 enzyme stimulated by secretin. Δ^9-THC (at either 1 or 30 μM) failed to alter the transition temperature or the activation energies (data not shown) when compared to the vehicle values in N4TG1 membranes.

The data were further analyzed for changes in enthalpy of activation ($\Delta H^\dagger$), entropy of activation ($\Delta S^\dagger$), and free energy of activation ($\Delta G^\dagger$) (Table 2). The changes in the transition temperature for benzyl alcohol and CBD were not accompanied by differences in $\Delta H^\dagger$ from the vehicle controls. These data are consistent with the findings that the activation energies for benzyl alcohol and CBD did not differ from control values, since the activation energy contains only an enthalpy component. Although the $\Delta G^\dagger$ values below the transition temperature for these agents were lower than controls, these alterations were not associated with a consistent alteration of the $\Delta H^\dagger$ or $\Delta S^\dagger$ for these two drugs.

Carbachol and Δ^9-THC, the agents that inhibit adenylate cyclase via G_i, showed a tendency to increase $\Delta G^\dagger$ with respect to control above the transition temperature. A decrease in the $\Delta H^\dagger$ with respect to control was also noted. The decreased $\Delta H^\dagger$ was countered by the tendency for a decreased $\Delta S^\dagger$ with respect to control. At temperatures below the break point of the Arrhenius plot, carbachol and Δ^9-THC did not alter significantly the $\Delta G^\dagger$ from control values. When compared with the control, carbachol produced a significant decrease in the $\Delta H^\dagger$ which was countered by a significant decrease in the $\Delta S^\dagger$. Decreases from control in $\Delta H^\dagger$ and $\Delta S^\dagger$ were also observed for both 1 and 30 μM Δ^9-THC.

DISCUSSION

Benzyl alcohol and CBD were able to increase adenylate cyclase activity and shift the break point of the Arrhenius plot to a lower temperature in neuroblastoma membranes. Increases in membrane fluidity have been reported to increase adenylate cyclase activity in a number of experimental systems [6, 7]. A break point in the Arrhenius plot of glucagon-stimulated adenylate cyclase correlates with a change in the fluidity of liver membranes as detected by ESR studies using a fatty acid spin-label probe [6, 30]. In those studies, benzyl alcohol increased adenylate cyclase activity at any given temperature by shifting the break point in the thermal activity curve similarly to what we report here for secretin-stimulated adenylate cyclase in neuroblastoma membranes. The mechanism for an influence of membrane fluidity on enzyme activity has not been fully explained [35, 36]. One possible explanation for the effects of increased membrane fluidity on adenylate cyclase could be that, as the gel-phase regions melt, there could be an increase in the size or composition of the fluid domain in which the adenylate cyclase proteins reside. The release into the fluid phase of lipids having a higher transition temperature [6, 35, 36] would result in an alteration of the lipid composition within the environment of the adenylate cyclase proteins. Several studies have indicated that hormone-stimulated adenylate cyclase is sensitive to specific lipid species [8–11]. Such a modification of activity may be subtle, as is the small increase in adenylate cyclase activity observed in the presence of membrane-fluidizing agents.

Such a mechanism would be consistent with what is currently known about the influence of cannabinoid drugs on neuronal membrane fluidity. It is probable that the lipid domains that are fluidized by can-

Table 2. Thermodynamic parameters for adenylate cyclase activity*

	303°K			285°K		
	$\Delta G^{\dagger}$ kcal/mol	$\Delta H^{\dagger}$ kcal/mol	$\Delta S^{\dagger}$ cal/mol/deg	$\Delta G^{\dagger}$ kcal/mol	$\Delta H^{\dagger}$ kcal/mol	$\Delta S^{\dagger}$ cal/mol/deg
Vehicle 1	1.07 ± 0.07	7.56 ± 1.78	21.4 ± 5.68	1.86 ± 0.13	20.8 ± 1.56	66.3 ± 5.07
Benzyl alcohol†	1.07 ± 0.05	7.55 ± 1.88	21.9 ± 6.73	1.40 ± 0.09‡	25.3 ± 3.74	83.8 ± 13.2
1 μM Δ^9-THC	3.33 ± 2.21	5.89 ± 0.48	15.9 ± 1.75	1.67 ± 0.09	18.9 ± 2.64	60.5 ± 9.27
1 mM Carbachol	1.28 ± 0.11§	6.68 ± 0.27	17.8 ± 0.93	1.86 ± 0.11	15.6 ± 0.29‡	48.2 ± 0.70‡
Vehicle 2	0.85 ± 0.07	9.04 ± 0.85	27.0 ± 2.64	1.85 ± 0.03	23.8 ± 2.29	76.9 ± 8.0
30 μM Δ^9-THC	1.07 ± 0.12§	6.32 ± 1.57§	17.4 ± 4.99§	1.80 ± 0.16	18.4 ± 2.15§	58.2 ± 7.28§
30 μM CBD	1.00 ± 0.05§	8.02 ± 1.04	23.2 ± 3.59	1.71 ± 0.02‡	21.9 ± 0.85	71.0 ± 3.00

* The data were analyzed from the same experiments represented in Table 1.

† The $\Delta G^{\dagger}$ for benzyl alcohol was calculated using the specific activity at the greatest temperature below the appearance of thermal inactivation.

‡ Different from vehicle at the $P < 0.05$ level of significance.

§ Different from vehicle at the $P < 0.2$ level of significance.

nabinoid drugs are different from those in which adenylate cyclase components reside. The fluorescence polarization data of Hillard *et al.* [24] using vesicles of varying rigidity and probes that localize preferentially in more fluid domains suggested that the cannabinoid drugs increase rather than decrease membrane order in more fluid domains. Cannabinoid compounds fluidized the more rigid membranes as detected by 1,6-diphenylhexatriene [24]. Since the proteins of the adenylate cyclase system would be expected to be excluded from rigid gel-phase domains [6], an increase in mobility of the lipids within these solid domains by cannabinoid drugs would not be expected to influence these transmembrane proteins directly. Of particular importance to the adenylate cyclase system, Hillard *et al.* [24] demonstrated that cannabinoid drugs have little effect on vesicles made from phosphatidylethanolamine plus phosphatidylserine, lipids that mimic the composition of the inner leaflet of the synaptic plasma membrane. This would suggest that the G-proteins associated with the inner membrane would not be influenced directly by lipid mobility changes induced by cannabinoid drugs.

The question arises as to why CBD but not Δ^9-THC altered the transition temperature of hormone-stimulated adenylate cyclase in neuroblastoma membranes. Differential responses to these two cannabinoid compounds have been observed under certain conditions in membrane fluidity studies. Hillard *et al.* [24] determined that, within synaptosomal membranes, Δ^9-THC at 3 μM decreases fluorescence polarization to a degree similar to 80 mM ethanol, but CBD does not. Calculations of solid state ^{2}H-NMR data by Makriyannis *et al.* [37] showed that Δ^9-THC orients with the long axis of the three ring structure almost orthogonal to the lipid bilayer chains allowing the phenolic hydroxyl group to face toward the polar side of the bilayer and the 5-carbon alkyl side chain to extend into the hydrophobic region of the bilayer. Because the structure of CBD differs from Δ^9-THC, possible differences in surface orientation or selectivity in interactions with certain lipid species may explain the differences reported here between Δ^9-THC and CBD in their modification of the transition temperature of adenylate cyclase activity.

The possibility also exists that agents such as benzyl alcohol and CBD, which slightly increase adenylate cyclase activity, may be acting by some mechanism other than by increasing membrane fluidity. The possibility that these compounds may interact directly with a protein in the enzyme complex has been suggested by others [30, 38]. Rabin and colleagues [39] have concluded that the stimulation of adenylate cyclase by short-chain aliphatic alcohols is not simply a consequence of fluidization of bulk membrane lipid. Hoffman and Tabakoff [40] have proposed that the interaction of ethanol with mouse striatal adenylate cyclase may be due to a direct interaction with a protein or proteins in the adenylate cyclase system. In those studies, 75 or 750 mM ethanol failed to affect either the break point of the Arrhenius plot or the activation energies of dopamine-stimulated adenylate cyclase. To explain the increased adenylate cyclase activity observed in the absence of an alteration in the phase transition, Hoffman and Tabakoff [40] proposed that an increase in $\Delta S^{\dagger}$ resulted from a stabilization by ethanol of the enzyme in a more reactive conformation. This would imply a direct protein–ethanol interaction rather than a modification of the lipid environment. Since the studies reported here demonstrate a clear decrease in the transition temperature associated with adenylate cyclase activity and no significant change in $\Delta S^{\dagger}$, the effects on membrane fluidization are favored.

Thermodynamic descriptions of hormone-regulated adenylate cyclase are sparse in the literature. Sinensky *et al.* [41] examined the adenylate cyclase of CHO-K1 cells and determined that the $\Delta H^{\dagger}$ increased by 1–3 kcal/mol in going from basal activity to prostaglandin E_1-stimulated activity. This increase was an average of 16% of the $\Delta H^{\dagger}$ for the basal state. The positive entropy change was greater in magnitude than the increased enthalpy change, indicating that the activation of adenylate cyclase by hormonal stimulators was entropy-driven [41]. The

net difference in $\Delta G^{\dagger}$ upon addition of the stimulatory hormone was a 0.5 kcal/mol decrease.

Some observations may be made from the thermodynamic data reported here regarding the regulation of adenylate cyclase by the inhibitory agonists carbachol and Δ^9-THC. Activation of the G_i pathway resulted in a decrease in $\Delta H^{\dagger}$ of 1–2 kcal/mol at temperatures above, and 2–5 kcal/mol at temperatures below, the break point in the Arrhenius plot. These changes represent a 20% decrease from $\Delta H^{\dagger}$ in the absence of inhibitory agonists. These decreases in enthalpy were countered by decreases in entropy that were of a smaller magnitude. The entropy change represented an approximate 20% decrease from the values obtained in the absence of inhibitory hormones. The net $\Delta G^{\dagger}$ was increased at physiologically relevant temperatures by 0.2 kcal/mol when the inhibitory pathway was activated by inhibitory agonists. These results suggest that, when the inhibitory pathway is activated, less disorder in the system occurs than in the presence of stimulatory hormones alone. The results of Sinensky *et al.* [41] indicated that stimulation of adenylate cyclase is associated with an increase in enthalpy which is overcome by an increase in the disorder of the system upon activation. The results reported here suggest that this action is opposed in the presence of hormonal inhibitors. This interpretation of the thermodynamic data is consistent with current models of adenylate cyclase regulation in which dissociation of G_i interferes with the ability of G_s to activate the catalytic protein [1].

In summary, membrane fluidity affected hormone-stimulated adenylate cyclase in neuroblastoma membranes as evidenced by the decrease in the transition temperature due to addition of benzyl alcohol. CBD evoked a similar response, suggesting that this cannabinoid drug may alter membrane fluidity as a mechanism to augment adenylate cyclase activity to a modest extent. No evidence exists for an effect of Δ^9-THC on the transition temperature, however. In contrast, Δ^9-THC behaved similarly to carbachol, both inhibiting enzyme activity via the G_i pathway to oppose the increased entropy change that resulted from activation by hormonal stimulators.

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