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Involvement of G_i in the Inhibition of Adenylate Cyclase by Cannabimimetic Drugs

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SUMMARY

The cellular mechanism of action of the cannabimimetic drugs is examined using cultured cells. In membranes from N18TG2 neuroblastoma cells and the neuroblastoma × glioma hybrid cells, NG108-15, the psychoactive cannabinoid drugs and their nantradol analogs could inhibit adenylate cyclase activity. This response was not observed in either the soluble adenylate cyclase from rat sperm or membrane-bound adenylate cyclases from C6 glioma or S49 lymphoma cells. This cellular selectivity provides further evidence for the existence of specific receptors for the cannabimimetic compounds. Receptor-mediated inhibition of adenylate cyclase requires the presence of a guanine nucleotide-binding protein complex, G_i. G_i can be functionally inactivated as a result of an ADP-ribosylation modification catalyzed by pertussis toxin. The present study demonstrates that pertussis toxin treatment of cells abolished the cannabimimetic response in intact cells and in membranes derived therefrom. The action of pertussis toxin required NAD^+ as substrate for *in vitro* modification of neuroblastoma membranes. Furthermore, pertussis toxin was able to catalyze the labeling of a neuroblastoma membrane protein *in vitro* using [^{32}P]NAD^+ under conditions similar to those by which attenuation of the cannabimimetic inhibition of adenylate cyclase could be demonstrated. This evidence demonstrates the requirement for a functional G_i in the action of cannabimimetic drugs.

The behavior of the psychoactive cannabinoid drugs has been shown to be dose dependent, pharmacologically selective, and stereospecific in man and a variety of animal models (1–6). This would suggest that these compounds act via a pharmacologically distinct receptor(s). However, until recently, study of the mechanism of action of these drugs has been hindered by the lack of a cellular model system in which dose-dependent, pharmacologically selective, and stereospecific effects of the cannabimimetic drugs could be demonstrated. We have demonstrated such properties for the inhibition of adenylate cyclase in a cloned line of neuroblastoma cells (7, 8). Thus, the mechanism of action of these drugs at the cellular level can be further investigated.

In previous work (8), it was shown that Δ^9-THC and its synthetic analog, desacetyllevonantradol, inhibited adenylate cyclase in a membrane preparation from neuroblastoma cells in a rapid and reversible manner. The inhibition was greatest at micromolar concentrations of Mg^{2+} or Mn^{2+}, and was attenuated or abolished as the divalent cation concentration was increased. The inhibition required at least 10-fold greater levels of GTP than were required for hormonal stimulation of the enzyme. Under certain conditions, the inhibition could be demonstrated using the nonhydrolyzable GTP analog, guanosine 5′-(β,γ-imino)triphosphate. These data are consistent with the mechanism of inhibition involving the GTP-binding protein complex associated with receptor-mediated inhibition of adenylate cyclase, referred to as G_i.

The present study provides additional evidence for the involvement of a specific receptor for the cannabimimetic drugs, in that the response is not universally observed in adenylate cyclase preparations from all cell types. Further evidence for the involvement of G_i is provided by the demonstration that pertussis toxin can attenuate or block the cannabimimetic response in both neuroblastoma cells and in membrane preparations.

Materials and Methods

Cyclic AMP production. Growth conditions for the cultured cells were as previously described (9, 10). For those experiments in which cells were exposed to pertussis toxin, flasks (175 cm^2) were grown until the cells were nearly confluent (2 or 3 days). The medium was changed and 100 ng/ml of pertussis toxin or vehicle were added 24 hr before harvesting. The cells were harvested with phosphate-buffered saline that contained 0.6 mM EDTA. Cells were washed twice by sedimentation in Gey's balanced salt solution and then resuspended at 2×10^6 cells/ml in Gey's solution containing 0.1 mg/ml of fatty acid-free bovine

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ABBREVIATIONS: THC, tetrahydrocannabinol; EDTA, ethylenediaminetetraacetic acid; RO20-1724, 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

serum albumin plus 0.1 mM RO20-1724. The cells were equilibrated for 30 min at 37° and then added in 400-μl aliquots to tubes that contained 100 μl of the appropriate drugs. After 4 min, the incubation was stopped by the addition of 50 μl of 500 mM sodium acetate, pH 4.5, and boiling. The particulate matter was sedimented, and cyclic AMP was determined in the supernatant by the method of Brostrom and Kon (11).

When adenylate cyclase was assayed, treated and control cells were harvested and membranes prepared as previously described (8) except that the sucrose density gradient sedimentation was omitted and the membranes were assayed immediately. Rat testes cytosol was prepared as described by Braun and Dods (12). The adenylate cyclase reaction was carried out exactly as previously published (8).

Pertussis toxin treatment of plasma membranes. The sucrose gradient-purified membrane preparation (750 μg of protein) was incubated in the presence or absence of 33 μg/ml of pertussis toxin. The reaction medium contained in a final volume of 300 μl: 25 mM Tris buffer, pH 7.2, 1 mM ATP, 10 mM thymidine, 1 mM EDTA, 2 mM $MgCl_2$, the nucleotide-regenerating system of 3 mM K^+ phosphoenolpyruvate, and 10 μg/ml of pyruvate kinase, and, where indicated, 2.5 mM NAD^+. After 30 min at 37°, the reaction was terminated by a 10-fold dilution of samples with ice-cold 20 mM Na HEPES, pH 8.0, containing 1 mM EDTA and 2 mM $MgCl_2$, followed by centrifugation at 100,000 × *g* for 5 min. The pellet was resuspended in the above dilution buffer and adenylate cyclase was assayed immediately.

SDS-PAGE. For the SDS-PAGE studies, the membrane preparation was treated as above except that the pertussis toxin had been freshly preactivated with 16.6 mM dithiothreitol at 37°. In addition to the components listed above, the incubation mixture also contained 10 μM *N*-α-*p*-tosyl-*l*-lysine chloromethylketone, 300 μM phenylmethylsulfonyl fluoride, 3.3 mM dithiothreitol, 2 μM GTP, and [^{32}P]NAD^+; the NAD^+ was present at 8 μM. Following incubation and sedimentation, the pellet was resuspended in 100 μl of gel sample buffer (1% SDS, 20 mM Na_2CO_3, 25 mM dithiothreitol, 80 mg/ml of bromphenol blue, 10% sucrose), heated at 100° for 1 min, and kept frozen until use for electrophoresis.

Aliquots of radiolabeled membranes in gel sample buffer (approximately 150 μg of protein) were subjected to SDS-PAGE on 1.5-mm-thick slab gels containing a 10% acrylamide separating gel and 6% stacking gel containing 0.1% SDS (13). Electrophoresis was performed for 5 hr at 26 mamp in Tris-HCl, pH 9.18. Gels were stained with 0.1% Coomassie blue. After destaining, gels were exposed to Kodak X-Omat film for 24–48 hr. Molecular weight estimations were by linear regression analysis using the following markers: carbonic anhydrase, 31,000; ovalbumin, 45,000; bovine serum albumin, 66,200; and phosphorylase *b*, 92,500.

Materials. The sources and handling of drugs have been documented (8, 10). Preliminary experiments were carried out using an initial supply of pertussis toxin generously provided by Dr. Eric Hewlett of University of Virginia. Subsequent studies were performed using pertussis toxin purchased from List Biological Laboratories. [^{32}P]NAD was obtained from ICN, and molecular weight markers were from Bio-Rad Laboratories.

Results

Cellular selectivity of the cannabimimetic inhibition of adenylate cyclase. The ability of cannabimimetic drugs to inhibit adenylate cyclase is not observed in all adenylate cyclase preparations. A soluble form of adenylate cyclase, found in cytoplasm from rat sperm (12), probably exists as a catalytic protein that is not regulated by guanine nucleotide-regulatory proteins. The evidence for this is that the enzyme is activated by Mn^{2+} but not by guanine nucleotides or by fluoride (12, 14), and interaction with detergent-solubilized G_s from mammalian sources is not observed (14). As shown in Fig. 1, no inhibition of this soluble enzyme occurred upon addition of cannabimimetic compounds to the assay mixture. Other laboratories have

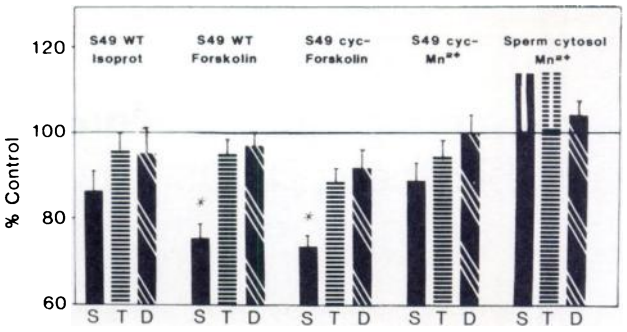

Fig. 1. Lack of cannabimimetic effects on several adenylate cyclase preparations. Adenylate cyclase activity was measured as described in the text except that $MgCl_2$ was 2 mM and ATP was 0.25 mM. The drug concentrations were 1 μM isoproterenol (plus 100 μM ascorbic acid), 30 μM forskolin, or 5 mM $MnCl_2$ as indicated. Within each group, inhibitory compounds were 5 μM somatostatin (*S*), 1 μM Δ^9-THC (*T*), or 1 μM desacetyllevonantradol (*D*). The data are expressed as a percentage of the adenylate cyclase activity measured in the absence of inhibitory compounds. Data are the means ± SE for three experiments. For sperm cytosol, the data show the mean and the range of two experiments. *, values statistically lower than control at $p < 0.02$ using the Student's *t* test.

demonstrated that sperm cytoplasmic adenylate cyclase is not activated by forskolin (15, 16). Our studies have confirmed this and have determined that cannabinoid drugs have no influence in the presence or absence of forskolin (data not shown). In a previous report, it was shown that cannabimimetic drugs did not alter the K_m for substrate of neuroblastoma adenylate cyclase (8). That finding, together with the failure to observe a cannabimimetic response in soluble sperm enzyme, implies that the catalytic protein of adenylate cyclase is not the target for these drugs.

The inhibition of adenylate cyclase by cannabimimetic drugs is not a characteristic of all membrane-bound adenylate cyclases. The S49 lymphoma cell has a well characterized adenylate cyclase that is stimulated by β-adrenergic compounds and inhibited by somatostatin (9, 14, 17). As shown in Fig. 1, the cannabimimetic agents did not inhibit receptor-stimulated or forskolin-activated adenylate cyclase in membranes from wild-type S49 cells under conditions in which somatostatin significantly inhibited the enzyme. The *cyc*$^-$ variant of S49 is devoid of a functional guanine nucleotide-binding protein that couples to stimulatory hormone receptors (G_s) (14). However, this variant cell retains a functional G_i subunit (17, 18). Adenylate cyclase in S49 *cyc*$^-$ membranes is active in the presence of Mn^{2+} and can be activated by forskolin (18). In confirmation of the work of Jakobs and Schultz (17), adenylate cyclase activity was inhibited by somatostatin in the presence of forskolin. However, no significant inhibition was produced by cannabimimetic agents (Fig. 1). The inhibition by somatostatin was attenuated by the presence of the activator Mn^{2+}, and the cannabimimetic response again was not observed. An uncoupled variant of S49 (*unc*), defective in the ability of G_s to interact with stimulatory receptors, also did not respond to Δ^9-THC or to desacetyllevonantradol (data not shown).

The NG108-15 cell, a hybrid of the N18TG2 neuroblastoma and the C6BU1 glioma (a subclone of C6), is one of the best characterized models of hormone-regulated inhibition of adenylate cyclase. Receptors for muscarinic cholinergic, α_2-adrenergic, and δ-opioid agonists are associated with decreased cyclic AMP accumulation in intact NG108-15 cells and inhi-

bition of adenylate cyclase in membrane fractions from these cells (see Refs. 19 and 20, and references contained therein). Shown in Table 1 is the inhibition of adenylate cyclase by carbachol and by morphine via the muscarinic and opioid receptors, respectively. This receptor-mediated inhibition was reversed by the antagonists atropine and naloxone at the two receptors, respectively. Although the N18TG2 cell parent possesses the neurohormone receptors for this inhibition, the expression of the response to cholinergic and opioid agonists was not as great as in the hybrid cell. This is a consistent finding in this laboratory and has been reported previously by Law *et al.* (20) for the opioid inhibition of adenylate cyclase. Both cell types express the inhibitory response to cannabimimetic agents. However, the response of the N18TG2 cell was consistently greater. Secretin was used as the stimulatory hormone in previous studies characterizing the cannabinoid response in N18TG2 cell membranes (7, 8). However, secretin is a poor stimulator of adenylate cyclase in membranes from NG108-15 (10). For comparative purposes, the present study was carried out using prostaglandin E_1 as the hormonal stimulator. However, this should not affect the activity of inhibitory hormones since the cannabimimetic inhibition in N18TG2 occurs to the same extent in the presence of either eicosanoid or peptide-stimulatory hormones (7).

The adenylate cyclase activity of C6 glioma cell membranes was not affected by cannabimimetic agents (Table 1). Experiments in this laboratory have failed to detect inhibition of C6 membrane adenylate cyclase in response to carbachol, somatostatin, or morphine (data not shown). However, a G_i component has been demonstrated in membranes from this cell, both by ADP-ribosylation and by enhancement of the β-adrenergic stimulation of adenylate cyclase after treatment with pertussis toxin (21).

Results presented here show that, for several cultured cell types, no effects of Δ^9-THC or desacetyllevonantradol could be detected. This demonstration that cannabimimetic inhibition is not universally observed in all plasma membrane adenylate cyclase systems provides further evidence consistent with the hypothesis that these drugs act via a pharmacologically specific receptor rather than by perturbations of membrane fluidity. Hillard and Bloom (22) attributed altered β-adrenergic receptor binding in a mouse cortex homogenate in the presence of 10 μM Δ^9-THC to changes in membrane structure induced by the lipophilic cannabinoid drug. The present results, however, indicate that concentrations of Δ^9-THC that maximally inhibit the enzyme in N18TG2 membranes do not have any influence on β-adrenergic stimulation of adenylate cyclase in S49 lymphoma or C6 glioma cell membranes.

Pertussis toxin blocks cannabimimetic response in intact neuroblastoma cells. Considerable evidence indicates that a toxin from *Bordetella pertussis* (also referred to as islet-activating protein) is able to block receptor-mediated inhibition of adenylate cyclase. Pertussis toxin is internalized by the cell and modified such that the "A" protein can catalyze the ADP-ribosylation of the alpha protein of G_i [see reviews by Gilman (23) and Ui *et al.* (24)]. Therefore, if cannabimimetic inhibition of adenylate cyclase requires the mediation of G_i, then the inhibition should be attenuated by treatment of the cells with pertussis toxin.

The studies performed to date have utilized membrane preparations from neuroblastoma cells. Prior to extensive studies of the inhibition of cyclic AMP accumulation in cells, we determined that the conditions used for these experiments did not alter the gross morphology or compromise the viability of the cells.[1] The intact cell response to cannabinoid and nantradol compounds is examined in the experiments shown in Fig. 2. Secretin produced a 6-fold increase in cyclic AMP accumulation in the presence of RO20-1724, a cyclic nucleotide phosphodiesterase inhibitor. The inhibition produced by Δ^9-THC was 38% when compared with the ethanol-containing control (Fig. 2A). In this experiment, the ethanol (2.2 mM) produced a stimulation of cyclic AMP accumulation, but this effect of ethanol was not reproducibly observed from experiment to experiment. Both cannabinol and cannabidiol produced less than a 15% decrease in cyclic AMP accumulation at a concentration maximally effective for Δ^9-THC. The inhibition by cannabichromene, which must be compared with the non-ethanol control, was less than 10%. Thus, the biologically inactive products of marihuana failed to significantly reduce the cyclic AMP accumulation in neuroblastoma cells. At concentrations greater than 1 μM, cannabidiol (Fig. 2B) and cannabinol (data not shown) increased cyclic AMP accumulation

[1] J. Dill and A. Howlett, unpublished observations.

TABLE 1

Effects of cannabimimetic drugs on neuroblastoma but not glioma cell membranes

The results are from a single experiment that is representative of three similar experiments. Drug concentrations were all 1 μM except carbachol (300 μM) and naloxone (10 μM).

Cell type	Adenylate cyclase (pmol/min/mg protein)					
	Basal	PGE_1	PGE_1 + carbachol	PGE_1 + morphine	PGE_1 + Δ^9 THC	PGE_1 + DALN[a]
N18TG2	23	211	172 (19%)[b]	171 (19%)	164 (22%)	106 (50%)
+Atropine	–[c]	220	220 (none)	–	173 (21%)	106 (52%)
+Naloxone	–	221	173 (22%)	210 (5%)	174 (21%)	105 (52%)
NG108-15	30	345	232 (33%)	211 (39%)	290 (16%)	276 (20%)
+Atropine	–	326	317 (3%)	–	290 (11%)	276 (16%)
+Naloxone	–	331	232 (30%)	359 (none)	293 (12%)	273 (18%)
	Basal	INE[d]			INE + Δ^9 THC	INE + DALN
C6[e]	25	45			45 (none)	43 (4%)

[a] DALN, desacetyllevonantradol.
[b] Numbers in parentheses, percentage of inhibition compared with the stimulatory hormone alone.
[c] –, not done.
[d] INE, isoproterenol.
[e] Membranes (39,000 × *g* pellet) were not further purified by sucrose density gradient sedimentation. C6 assay tubes all contained 100 μM ascorbate.

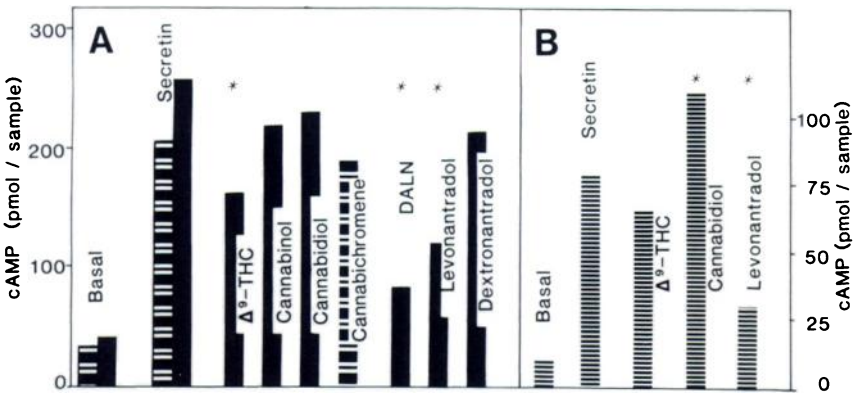

Fig. 2. Cyclic AMP accumulation in the presence of cannabinoid drugs in N18TG2 cells. Neuroblastoma cells were harvested and cyclic AMP content was determined in the presence of the indicated drugs. Except for the basal points, all tubes contained 1 μM secretin. The cannabinoid and nantradol compounds were present at 1 μM in A and 3 μM in B. In A, the *solid bars* contain the same concentration of ethanol vehicle (0.01%). The *striped bars* represent the absence of vehicle or, in the case of cannabichromene, ≤0.001% ethanol. The data are presented as the mean of three sample tubes (each assayed for cyclic AMP in duplicate); the average coefficient of variation [100 (SD)/mean] for the data from these experiments was 20%. *, values that are statistically different from the secretin control at $p < 0.05$ using the Student's *t* test. *DALN*, desacetyllevonantradol.

above the level of stimulation by the appropriate ethanol controls. In some experiments, the inhibition by Δ^9-THC was also attenuated at concentrations greater than 1 μM. This effect might be similar to the ability of a low concentration of ethanol to stimulate cyclic AMP accumulation (25), an effect perhaps due to membrane fluidity influences on adenylate cyclase activity (26). For the nantradol series of cannabinoid analogs, desacetyllevonantradol and levonantradol produced a 69% and a 59% inhibition of cyclic AMP accumulation, respectively (Fig. 2A). The biologically less active isomer, dextronantradol, produced only a 17% inhibition at the same concentration. This pharmacological profile is consistent with a receptor-mediated, stereospecific response in intact neuroblastoma cells.

The experiment shown in Fig. 3 was designed to determine whether pertussis toxin could alter the cannabimimetic response in intact neuroblastoma cells. Incubation of the cells with 100 ng/ml of pertussis toxin for 24 hr was sufficient to totally block the responses to Δ^9-THC and to desacetyllevonantradol. Treatment with pertussis toxin also blocked the inhibition by carbachol at the muscarinic cholinergic receptor. The basal accumulation of cyclic AMP was higher in pertussis toxin-treated cells. This increase has been observed in other cell types (24) and may represent the removal of an inhibitory influence of G_i on the basal adenylate cyclase activity.

The effect of pertussis toxin treatment was maintained after disruption of the cells and sedimentation of a membrane fraction (Table 2). Basal and secretin-stimulated adenylate cyclase activities were increased by 30% and 55%, respectively, in membranes from pertussis toxin-treated cells. The inhibitions of adenylate cyclase activity by the cannabimimetic compounds and by carbachol were significantly attenuated. Only the inhibition by desacetyllevonantradol was significantly different from the secretin-stimulated control in the membranes from pertussis toxin-treated cells.

ADP-Ribosylation of a membrane protein associated with attenuation of the cannabimimetic response. Experiments were performed to demonstrate that the effects of pertussis toxin on cyclic AMP accumulation can be attributed to a modification of a membrane protein. A plasma membrane fraction was treated with pertussis toxin and its substrate, NAD^+, and adenylate cyclase activity was assayed in the washed membranes (Fig. 4). The adenylate cyclase suffered a loss of activity as a result of such treatment; the secretin-stimulated activity was only about one-half that of unincubated control membranes (not shown). However, inhibition by carbachol and the cannabimimetic drugs was not altered by incubation at 37° under these conditions. The addition of pertussis toxin plus NAD^+ in the incubation resulted in the attenuation of drug-mediated inhibition of activity. Incubation with either toxin or substrate alone failed to cause a change in inhibition in response to carbachol or desacetyllevonantradol, and resulted in only a partial reversal of the inhibition in response to Δ^9-THC. These data demonstrate that both toxin and substrate are required in order to block the inhibitory response in plasma membranes.

A plasma membrane protein is labeled by [^{32}P]NAD in the presence of pertussis toxin. As in shown in Fig. 5, a protein having an apparent molecular weight of 39,000–41,000 was

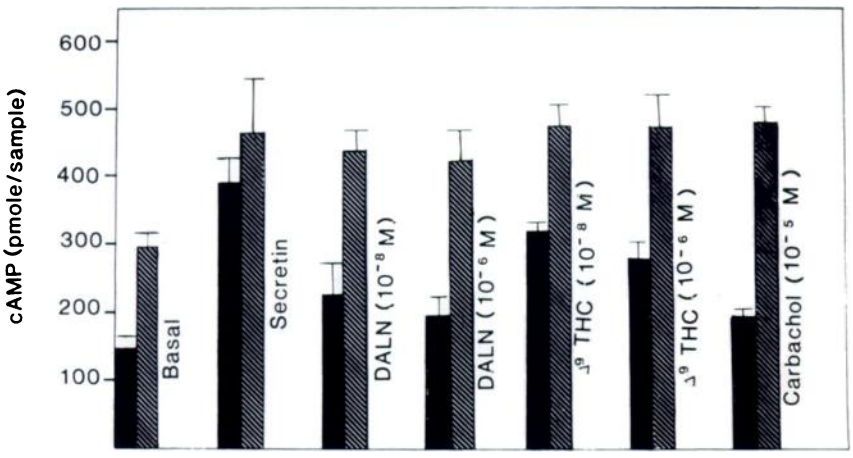

Fig. 3. Pertussis toxin effects on the inhibition of cyclic AMP accumulation in intact cells. Cells were treated without (*solid bars*) or with pertussis toxin (*hatched bars*), harvested, and tested for cyclic AMP accumulation in response to the indicated drugs. Except for the basal points, all samples contained 1 μM secretin. The data are presented as the mean ± SE of three sample tubes (each assayed for cyclic AMP in duplicate). *DALN*, desacetyllevonantradol.

TABLE 2

Inhibition of adenylate cyclase in membranes from pertussis toxin-treated neuroblastoma cells

Adenylate cyclase activity was determined (in triplicate) in membranes prepared from three separate flasks of cells treated with 100 ng/ml of pertussis toxin or control for 24 hr. The data are the mean ± SE (n = 3) and the numbers in parentheses are the percentage inhibition. This experiment is representative of two.

Assay addition	Adenylate cyclase	
	Control	Pertussis Toxin-Treated
	pmol/min/mg	
	12 ± 0.9	16 ± 1.7
Secretin 1 μM	33 ± 1.5	51 ± 2.5
+300 μM Carbachol	25 ± 2.0 (25%)[a]	52 ± 3.8 (none)
+1 μM Δ⁹-THC	28 ± 1.7 (14%)[a]	48 ± 2.1 (5%)
+300 nM DALN[b]	18 ± 1.1 (44%)[a]	45 ± 3.0 (11%)[a]

[a] Significantly lower than control at $p < 0.05$ using the Student's t test.
[b] DALN, desacetyllevonantradol.

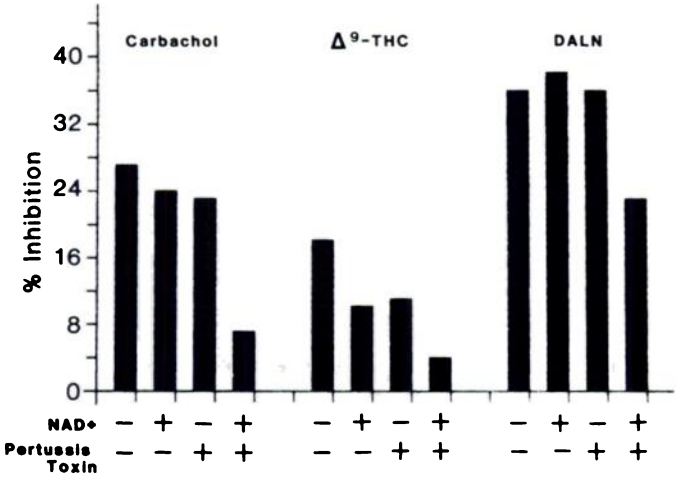

Fig. 4. Attenuation of adenylate cyclase inhibition by pertussis toxin and NAD^+ in neuroblastoma membranes. Plasma membranes were incubated as described in the text, with or without pertussis toxin, in the presence or absence of NAD^+ as indicated. Adenylate cyclase activity was determined in the presence of 0.8 μM secretin (88 pmol/min/mg) without or with 300 μM carbachol, 1 μM Δ⁹-THC, or 1 μM desacetyllevonantradol (*DALN*). This experiment is representative of four experiments.

specifically labeled by the toxin. In data not shown, cholera toxin labeled a protein having an apparent molecular weight of 45,000. Thus, a protein can be identified that is associated with the pertussis toxin-mediated attenuation of the cannabimimetic response in membranes. The labeling of a high molecular weight band (>100,000) in the absence of pertussis toxin is readily apparent. A similar band was reported by Gill and Meren (27) in pigeon erythrocyte ghosts and also appears in the ADP-ribosylation autoradiographs published by Burns *et al.* (28) for NG108-15 hybrid cell and by the laboratory of Ui (21) for C6 glioma cell membranes. An endogenous ADP-ribosyltransferase and its substrate may be present on the neuroblastoma cell membranes. An ADP-ribosyltransferase, which labels numerous proteins and guanidino compounds, has been isolated from turkey erythrocytes (29). However, the role of this vertebrate enzyme in the possible regulation of cellular functions has not been established (29).

The specific ADP-ribosylation by pertussis toxin of the protein band shown in Fig. 5 occurs in the intact cell in parallel with the loss of receptor-mediated inhibition of adenylate cyclase. This is demonstrated in Fig. 6 for desacetyllevonantradol.

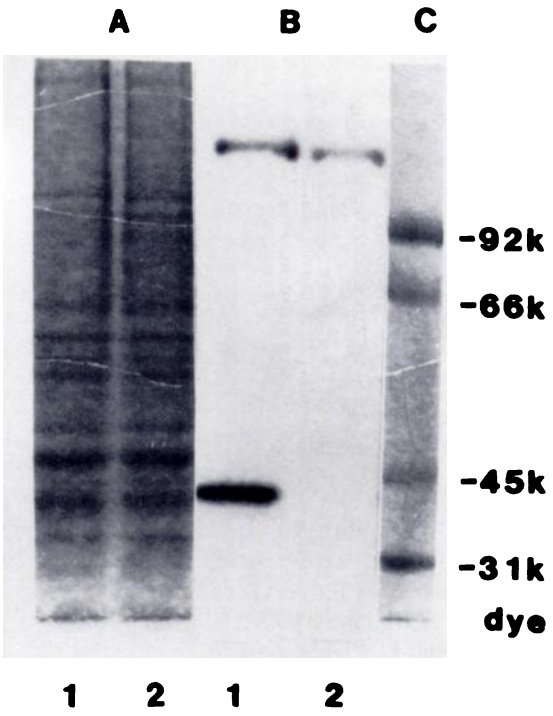

Fig. 5. Pertussis toxin labeling of a protein in neuroblastoma membranes. Membranes prepared from N18TG2 cells were incubated with [^{32}P]NAD as described in the text in the presence (*lanes 1*) or absence (*lanes 2*) of pertussis toxin. A. Coomasie blue-stained gel; B. autoradiograph; C, molecular weight markers.

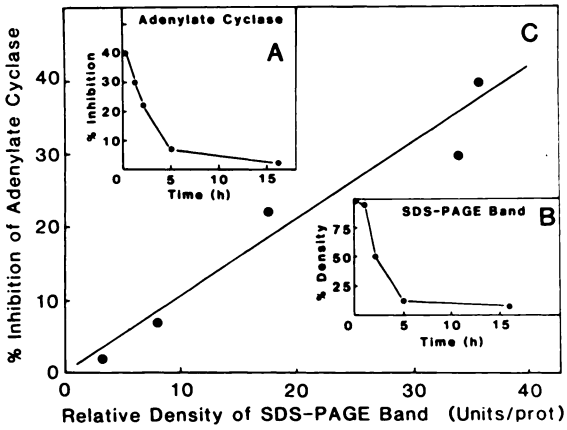

Fig. 6. Pertussis toxin-specific ADP-ribosylation coincident with the attenuation of cannabimimetic inhibition of adenylate cyclase. Duplicate flasks of neuroblastoma cells were incubated with 50 ng/ml of pertussis toxin for the indicated times. A. Cells were harvested and membranes prepared for immediate adenylate cyclase assay using conditions described in Table 2. B. Aliquots of the same membranes were treated with pertussis toxin plus [^{32}P]NAD as described for Fig. 5. Density of the bands from the SDS-PAGE autoradiographs was quantitated using an LKB2202 Ultroscan laser densitometer measuring transmittance at 632.8 nm. The area under the peak is in arbitrary units linear with transmittance, and has been corrected for total protein applied to each band. C. Data from A are correlated with data from B. This experiment was repeated with similar results.

Fig. 6A shows that the attenuation of the inhibition of adenylate cyclase was complete by 5 hr and was not reversed during the continued incubation with the toxin. Pertussis toxin-mediated ADP-ribosylation using intracellular NAD^+ precluded

the subsequent labeling of that protein by pertussis toxin plus [^{32}P]NAD$^+$ in membranes isolated from those cells (Fig. 6B). The time course of intracellular ADP-ribosylation is coincident with the loss of the cannabimimetic effects on adenylate cyclase (Fig. 6C). Identical results occurred for muscarinic inhibition of adenylate cyclase (not shown).

Discussion

Results presented in this communication demonstrate cellular selectivity of the cannabimimetic inhibition of adenylate cyclase. If the response to these agents had been the result of a direct interaction with the catalytic subunit of adenylate cyclase, then one would have expected to observe the inhibition in enzyme preparations from nearly all sources. Furthermore, if the cannabimimetic effects were due to membrane lipid fluidity changes resulting in inefficient receptor-effector interactions as postulated by others (22), then similar effects should be observed in membrane-bound adenylate cyclase complexes from a variety of cell types. The failure of a number of adenylate cyclase preparations to respond to cannabimimetic drugs, in addition to the previously demonstrated kinetics, dose dependence, pharmacological selectivity, and stereospecificity (7, 8), implicates a receptor for the cannabimimetic drugs.

In other systems, receptor-mediated inhibition of adenylate cyclase results from an interaction of the hormone-receptor complex with a guanine nucleotide-binding protein complex, G_i, and subsequent regulation of the catalytic subunit of the enzyme. The present study has investigated the requirement for G_i for the cannabimimetic inhibition. Pertussis toxin has been used as a tool in these experiments because of its ability to block the action of G_i. Pertussis toxin treatment of the cells was able to block the inhibition of cyclic AMP accumulation in intact cells caused by the cannabimimetic drugs and also by carbachol, a muscarinic receptor agonist. The pertussis toxin effect on adenylate cyclase regulation in intact cells was retained in membrane preparations from treated cells. The stability of this effect would be consistent with the covalent modification of the G_i α-protein. Such a modification was demonstrated by the requirement for the substrate, NAD$^+$, for the effectiveness of pertussis toxin treatment of purified membranes. Using conditions demonstrated to effectively attenuate the cannabimimetic effect on adenylate cyclase, pertussis toxin was able to catalyze the ADP-ribosylation of a protein band in the apparent molecular weight range of 39,000–41,000 on SDS-PAGE. These data clearly demonstrate a role for G_i in the molecular mechanism of the cannabimimetic drugs.

The protein that has been ADP-ribosylated is defined as the α-subunit of G_i, based on the functional activity to mediate adenylate cyclase inhibition. Other proteins have been shown to be modified by pertussis toxin, however (23). Recently, Sternweis and Robishaw (30) have demonstrated that a protein found in brain, referred to as G_o, is modified by pertussis toxin. However, no functional interaction of G_o with adenylate cyclase has been reported, and recent data support the notion that guanine nucleotide-regulatory proteins may not be functionally interchangeable (31). Other hormonally mediated functions have recently been shown to be blocked by pertussis toxin treatment of cells. These actions include calcium mobilization and arachidonic acid release in neutrophils (32, 33) and 3T3 fibroblasts (34), and cyclic GMP production in NG108-15 cells (35). These receptor-mediated processes may be coupled to G_i or to some other protein modified by pertussis toxin. Whether such functions are regulated by the same receptors that interact with G_i to inhibit adenylate cyclase is speculative. Cannabinoid compounds have been associated with arachidonic acid release and metabolism in human lung fibroblasts (36) and mouse peritoneal macrophages (37). It will be of interest to determine whether these latter cannabinoid effects are also mediated by a G protein.

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