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SUPPRESSION OF THE HUMORAL IMMUNE RESPONSE BY CANNABINOIDS IS PARTIALLY MEDIATED THROUGH INHIBITION OF ADENYLATE CYCLASE BY A PERTUSSIS TOXIN-SENSITIVE G-PROTEIN COUPLED MECHANISM

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Abstract—Cannabinoid compounds, including the major psychoactive component of marihuana, Δ^9-tetrahydrocannabinol (Δ^9-THC), have been widely established as being inhibitory on a broad array of humoral and cell-mediated immune responses. The presence of cannabinoid receptors has been identified recently on mouse spleen cells, which possess structural and functional characteristics similar to those of the G-protein coupled cannabinoid receptor originally identified in rat brain. These findings, together with those demonstrating that Δ^9-THC inhibits adenylate cyclase in splenocytes, strongly suggest that certain aspects of immune inhibition by cannabinoids may be mediated through a cannabinoid receptor-associated mechanism. The objective of the present studies was to determine whether inhibition of adenylate cyclase is relevant to mouse spleen cell immune function and, if so, whether this inhibition is mediated through a G_i-protein coupled mechanism as previously described in neuronal tissue. Spleen cell activation by the phorbol ester phorbol-12-myristate-13-acetate (PMA), plus the calcium ionophore ionomycin, produced a rapid but transient increase in cytosolic cAMP, which was inhibited completely by immunosuppressive concentrations of Δ^9-THC (22 μM) and the synthetic bicyclic cannabinoid CP-55940 (5.2 μM), which produced no effect on cell viability. Inhibition by cannabinoids of lymphocyte proliferative responses to PMA plus ionomycin and the sheep erythrocyte (sRBC) IgM antibody-forming cell (AFC) response, was abrogated completely by low concentrations of dibutyryl-cAMP (10–100 μM). Inhibition of the sRBC AFC response by both Δ^9-THC (22 μM) and CP-55940 (5.2 μM) was also abrogated by preincubation of splenocytes for 24 hr with pertussis toxin (0.1–100 ng/mL). Pertussis toxin pretreatment of spleen cells was also found to directly abrogate cannabinoid inhibition of adenylate cyclase, as measured by forskolin-stimulated accumulation of intracellular cAMP. These results indicate that inhibition of the sRBC AFC response by cannabinoids is mediated, at least in part, by inhibition of adenylate cyclase through a pertussis toxin-sensitive G_i-protein coupled cannabinoid receptor. Additionally, these studies further support the premise that cAMP is an important mediator of lymphocyte activation.

Key words: cannabinoids; immunosuppression; pertussis toxin-sensitive; adenylate cyclase/cAMP signal transduction

The mechanism by which cannabinoid compounds produce their broad spectrum of physiologic effects has been attributed historically to disruption of cell membrane processes through nonspecific intercalation of these highly lipophilic molecules into the lipid bilayer. In contrast to this view, a number of compelling lines of evidence have been forthcoming from studies primarily using neuronal tissue preparations and CNS-associated responses that implicate the role of a receptor-mediated mechanism for cannabinoid-induced effects. First, cannabinoids display stereoselective activity [1–6], a hallmark of receptor-mediated mechanisms. Second, cannabinoid receptor radioligands exhibit a high degree of specific binding to neuronal tissue preparations [2, 5, 7, 8]. Third, cannabinoids markedly inhibit adenylate cyclase in neuronal tissue preparations [9–13]. The recent isolation and cloning of a G-protein coupled cannabinoid receptor (CB1) from a rat brain cDNA library, taken together with previous indirect evidence, confirm the existence of cannabinoid receptors in association with the CNS [4]. In addition to direct effects on the CNS, cannabinoids have also been found to produce a variety of changes in peripheral non-CNS tissues of which the best characterized to date have been the testis and immune system. Interestingly, cannabinoid receptor mRNA has been identified in both tissues.

Recent studies in our laboratory have focused on characterizing the mechanisms by which cannabinoids modulate immunocompetence. Various animal and

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cell culture model systems have been utilized in past investigations to demonstrate that cannabinoids markedly inhibit humoral, cell-mediated and innate immune responses [reviewed in Ref. 14]. Although the mechanism responsible for these effects on immunocompetence has been elusive, recent identification of functional cannabinoid receptors on mouse spleen cells represents additional evidence suggesting that cannabinoid physiologic effects are, at least in part, mediated through a highly specific receptor-associated mechanism [15]. This conclusion was based on the following observations: demonstration by the synthetic cannabinoid receptor ligand [^{3}H]CP-55940* of a high degree of specific and saturable binding (greater than 60%) to mouse splenocytes exhibiting a K_d in the picomolar range; stereoselective inhibition of IgM AFC response using a variety of cannabinoid enantiomers including CP-55940/CP-56667; and the detection of cannabinoid receptor mRNA transcripts by RT-PCR [15]. The role of cannabinoid receptors in immune modulation is further supported by the fact that Δ^9-THC markedly inhibits adenylate cyclase activity in splenocytes [16], the same signal transduction pathway by which signalling through the cannabinoid receptor has been shown to occur in neuronal tissue preparation. Recently, further evidence has been forthcoming supporting the expression of cannabinoid receptors on lymphoid cells. Bouaboula and coworkers [17] reported the expression of cannabinoid receptors and CB1 mRNA transcripts on a variety of human leukocyte cell lines, as well as in primary human lymphoid cells. Most recently, Munro and coworkers [18] identified a cannabinoid receptor subtype, CB2, in the marginal zone of human spleen, which exhibits approximately 44% homology with the cannabinoid receptor originally identified in brain. Interestingly, the degree of homology between these two receptors increases to approximately 68% for those transmembrane residues proposed to confer ligand specificity. Radioligand binding analysis demonstrated that classic cannabinoid receptor ligands exhibit similar binding affinity for CB2 as previously reported for the brain receptor, CB1, with the exception of cannabinol, which has a higher affinity for the CB2. Interestingly, this novel peripheral cannabinoid receptor was not detected in brain tissue preparations.

Although the existence of cannabinoid receptors on lymphoid cells has been widely established, the relative distribution of CB1 and CB2 on lymphoid cells is unclear. Furthermore, it is also unclear how changes in signalling via the adenylate cyclase/cAMP second messenger pathway following cannabinoid ligand–receptor interactions alter normal physiologic processes. The implication that inhibition of adenylate cyclase by cannabinoids in mouse spleen cells may represent a critical biochemical event in mediating immune suppression by cannabimimetic agents is contrary to a generally held view that the adenylate cyclase/cAMP second messenger system is responsible for down-regulating immunological responses [see review in Ref. 19]. However, results from the present studies strongly suggest that the cAMP-mediated signal transduction pathway is critical to lymphocyte function. The objective of the present studies was to determine whether inhibition of adenylate cyclase by cannabimimetic agents is functionally relevant for lymphocyte function as measured by the sRBC AFC response and, if so, whether this inhibition is mediated through a G_i-protein coupled mechanism.

MATERIALS AND METHODS

Chemicals. Δ^9-THC was provided by the National Institute on Drug Abuse. CP-55940 and CP-56667 were gifts from Dr. Lawrence Melvin (Pfizer, Inc., Groton, CT).

Mice. Virus-free female B6C3F1 mice, 5–6 weeks of age, were purchased from the Frederick Cancer Research Center. On arrival, mice were randomized, transferred to plastic cages containing saw dust bedding (4 mice per cage), and quarantined for 1 week. Mice were given food (Purina Certified Laboratory Chow) and water *ad lib.* and were not used for experimentation until their body weight was 17–20 g. Animal-holding rooms were kept at 21–24° and 40–60% relative humidity with a 12-hr light/dark cycle.

cAMP determinations. Single spleen cell suspensions were prepared from naive female B6C3F1 mice. The cells were washed once with RPMI 1640 and centrifuged at 800 *g* for 10 min to form a pellet. EBSS without HEPES containing 5% NBCS at 1 mL/spleen in addition to Gey's solution was added to the cell pellet. The solution was swirled on ice for 5 min to lyse red blood cells. The remaining intact spleen cells were pelleted by centrifugation at 1600 *g* for 15 min, and the supernatant containing the lysates was discarded. The cells were washed twice in EBSS without HEPES buffer and adjusted to 1×10^7 cells/mL in RPMI containing 1% NBCS. These cells were then used for either temporal addition studies with PMA and ionomycin or to study the pertussis toxin sensitivity of the cAMP response. For the temporal addition studies, 1-mL aliquots of the isolated cells were transferred to 12×75 mL glass tubes, and 100 μM IBMX (Sigma, St. Louis, MO) was added to all of the cell treatment groups with the exception of appropriate controls (i.e. naive, vehicle). Following a 10-min incubation at 37°, cells were treated with either vehicle (1% DMSO), Δ^9-THC (22 μM) or CP-55940 (5.2 μM) and incubated for an additional 10 min at 37°. The cell preparations were then stimulated with PMA (80 nM) and ionomycin (1 μM) for 0.5 to 10 min. In the second series of cAMP experiments, the cells were treated with various concentrations of pertussis toxin (0.1 to 100 ng/mL) for 24 hr at 37°. Aliquots 1 mL of both the pertussis toxin-treated and

* Abbreviations: Δ^9-THC, Δ^9-tetrahydrocannabinol; sRBC, sheep erythrocytes; G-protein, guanine-nucleotide-binding protein; PMA, phorbol-12-myristate-13-acetate; Bt_2cAMP, dibutyryl-cAMP; RPMI 1640, Roswell Park Memorial Institute 1640; AFC, antibody-forming cell; NBCS, new born calf serum; EBSS, Earle's balanced salt solution; CP-55940/CP-56667, −/+ *cis*-3-[2-hydroxy-4-(1,1- dimethylheptyl)phenyl]- *trans*- 4- (3- hydroxypropyl)-cyclohexanol; IBMX, 3-isobutyl-1-methylxanthine; and RT-PCR, reverse transcription-polymerase chain reaction.

untreated cell suspensions were transferred to glass tubes followed by the addition of 100 μM IBMX to all of the cell treatment groups with the exception of appropriate controls (i.e. naive, vehicle and forskolin alone). Following a 10-min incubation at 37°, cells were treated with either vehicle (1% DMSO), Δ^9-THC (22 μM) or CP-55940 (5.2 μM) and incubated for an additional 5 min at 37°. The cell preparation was then stimulated with 50 μM forskolin (Sigma) for 15 min. After the stimulation with either PMA + ionomycin or forskolin, adenylate cyclase was inactivated by the addition of the cAMP extraction buffer: acidic ethanol (1 mL 1 of N HCl/100 mL EtOH). The cells were subsequently disrupted by sonication to facilitate the release of intracellular cAMP into the extraction buffer. The solution containing the cell lysate was centrifuged at 1600 g for 15 min to remove any remaining cell fragments, and the supernatants were collected and lyophilized. The samples were stored at −20° prior to quantitation of cAMP. Aliquots of reconstituted lyophilized cell lysates were quantitated for cAMP using a cAMP assay kit (Diagnostic Products Inc., Los Angeles, CA). This method is based on the competition between unlabeled cAMP and a fixed quantity of ^{3}H-labeled cAMP for binding to a protein that has a high specificity and affinity for cAMP, which mimics the regulatory subunit of protein kinase A. The amount of the ^{3}H-labeled cAMP protein complex formed is inversely related to the amount of unlabeled cAMP present in the assay sample. The concentration of cAMP in test samples was determined by comparison with a linear standard curve.

In vitro *antibody assays*. Spleens from untreated mice were isolated aseptically and made into a single cell suspension. The spleen cell suspension was adjusted to 1.0×10^7 cells/mL in RPMI 1640 supplemented with 5% NBCS (Hyclone, Logan, UT), 2 mM L-glutamine, antibiotic-antimycotic (100 units penicillin, 100 μg streptomycin and 0.25 μg fungizone/mL) (GIBCO, Grand Island, NY), and 5×10^{-5} M 2-mercaptoethanol (complete RPMI medium). When the effects of pertussis toxin were being studied, spleen cells were incubated at 37° for 24 hr in the presence or absence (control cells) of pertussis toxin (0.1 to 100 ng/mL). Following the preincubation step, cells were washed free of pertussis toxin, resuspended in complete RPMI at a density of 1.0×10^7 cells/mL, and transferred in 500-μL aliquots to a 48-well Costar culture plate (Cambridge, MA). Cultures were set up in quadruplicate for each treatment group. Δ^9-THC (22 μM) or CP-55940 (5.2 μM) was added directly in 5 μL of vehicle (DMSO, 0.01% final culture concentration) to the respective wells of the 48-well culture plate just prior to antigen sensitization. For the Bt_2cAMP studies, Bt_2cAMP (10–250 μM) was added directly to spleen cell cultures immediately after cannabinoid treatment and before antigen sensitization. Each well was sensitized with 6.5×10^6sRBC and cultured for 5 days in a Bellco stainless steel tissue culture chamber pressurized to 6.0 psi with a gas mixture consisting of 10% O_2, 7% CO_2 and 83% N_2. The culture chamber was maintained continuously rocking, on a rocker platform, for the duration of the culture period (i.e. 5 days). Enumeration of the AFC response was performed as previously described [20]. Briefly, spleen cells were resuspended in each well of the 48-well culture plate. A 50-μL aliquot of cell suspension was removed from each well and added to a 12 × 75 mm heated glass culture tube containing 400 μL of 0.5% melted agar (DIFCO, Detroit, MI) solution in EBSS and 0.05% DEAE-Dextran (Pharmacia, Piscataway, NJ). Additionally, each agar tube received 25 μL of guinea pig complement and 25 μL of indicator sRBC. The tubes were immediately vortex mixed, a 200-μL aliquot of the mixture was transferred to a 100 × 15 mm petri dish, and the agar solution was covered with a 25 × 50 mm microscope cover slip. Once the agar solidified, the petri dishes were incubated at 37° for 3 hr. Following the 3-hr incubation, the AFC were enumerated at 6.5 magnification using a Bellco plaque viewer. During the 3-hr incubation period, the number of spleen cells per well and viability (described below in "Pronase determination of viability") were determined using a Coulter Counter. Results from quadruplicate cultures were expressed as the mean AFC/10^6 recovered splenocytes ± SEM.

Pronase determination of viability. Aliquots of spleen cell suspensions were incubated with an equal volume (100 μL) of pronase (5 mg/mL) (Calbiochem-Behring Corp., San Diego, CA) for 10 min at 37°. Following the incubation, the splenocyte solution was diluted with 10 mL Isoton® (Coulter, Addison, NJ), counted on a Coulter counter, and compared with a 100-μL aliquot of the same test sample of

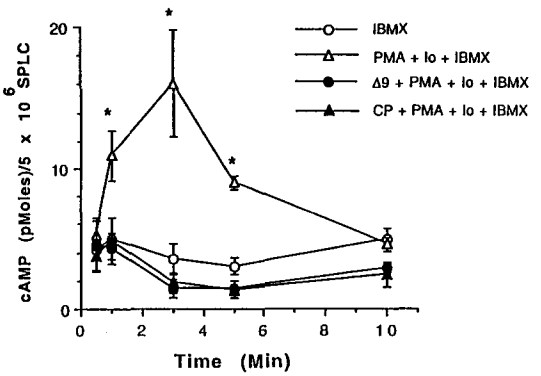

Fig. 1. Inhibition of adenylate cyclase in PMA plus ionomycin-stimulated mouse splenocytes by Δ^9-THC and CP-55940. Spleens from naive female B6C3F1 mice were isolated and made into a single cell suspension. Splenocytes were washed and adjusted to 5×10^6 cells/mL and transferred in 1-mL aliquots to 12 × 75 mm glass tubes. Spleen cells were incubated with 100 μM IBMX for 10 min and then treated with either Δ^9-THC (22 μM) or CP-55940 (5.2 μM) for 10 min followed by a 0.5 to 10-min stimulation with PMA (80 nM) + ionomycin (1 μM). Intracellular cAMP concentrations (pmol) are expressed as the means ± SEM of triplicate samples, as determined for each group, from one of three representative experiments. Key: (*) $P < 0.05$ as compared with the IBMX control group (determined by Dunnett's t-test).

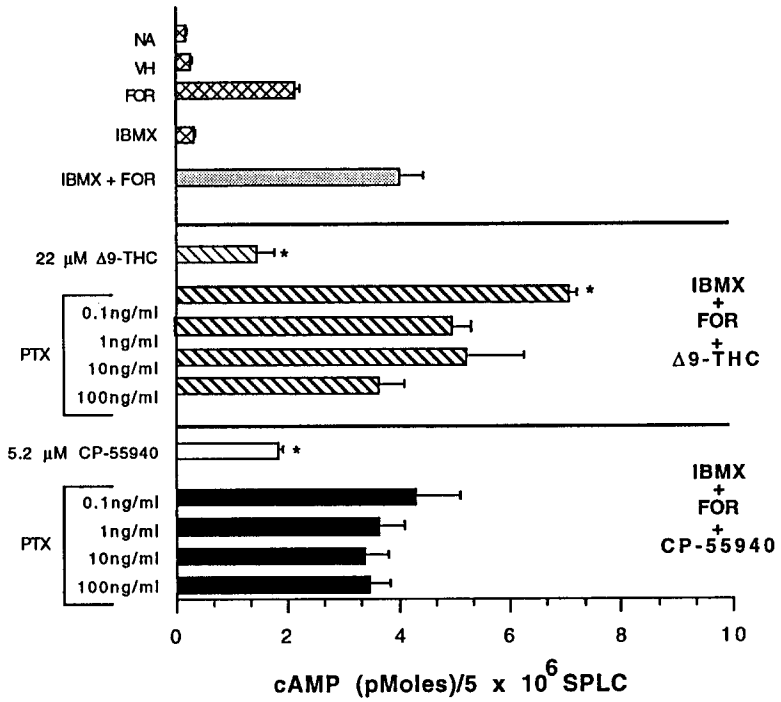

Fig. 2. Reversal of Δ^9-THC- and CP-55940-mediated inhibition of adenylate cyclase by pertussis toxin. Spleens from naive (NA) female B6C3F1 mice were isolated and made into a single cell suspension. Splenocytes were washed and adjusted to 5×10^6 cells/mL and aliquoted into 15-mL polypropylene tubes. Both naive and vehicle groups were left untreated for 18 hr at 37°, while the treatment groups were incubated with pertussis toxin (0.1 to 100 ng/mL) for 18 hr at 37°. Cells were transferred in 1-mL aliquots to 12 × 75 mm glass tubes. Spleen cells were incubated with 100 μM IBMX for 10 min and then treated with either vehicle (VH; 0.01% DMSO), Δ^9-THC or CP-55940 for 5 min followed by a 15-min stimulation with forskolin. Intracellular cAMP concentrations (pmol) are expressed as the means ± SEM of triplicate samples, as determined for each group, from one of three representative experiments. Pertussis toxin (10 ng/mL) ± IBMX + forskolin control treatment group = 5.9 ± 1.2 pmol cAMP/5 × 10^6 spleen cells. Key: (*) $P < 0.05$ as compared with the IBMX + forskolin control group (determined by Dunnett's *t*-test).

splenocytes without pronase. The percent viability = (cell counts with pronase/cell counts without pronase) × 100.

Lymphoproliferation with PMA plus ionomycin. Splenocytes were isolated aseptically from untreated mice and adjusted to 5×10^6 cells/mL in complete RPMI 1640 and transferred in 200-μL aliquots to a 96-well flat bottom microtiter plate (Costar, Cambridge, MA). Quadruplicate cultures were treated with either vehicle (0.01% DMSO), Δ^9-THC (22 μM) or CP-55940 (5.2 μM). The splenocytes were treated with PMA (80 nM) and ionomycin (1 μM) and incubated at 37° and 5% CO_2 for 72 hr. Splenocytes were pulsed with 1 μCi/well of [^{3}H]-thymidine for the last 16 hr of culture. Tritium incorporation was measured by scintillation counting.

Statistical analysis of data. The mean ± SEM was determined for each treatment group of a given experiment. The homogeneity of the results was determined using Bartlett's test for homogeneity [21]. Homogeneous data were evaluated by a parametric analysis of variance. When significant differences occurred, treatment groups were compared with the vehicle controls using a Dunnett's two-tailed *t*-test [22]. Nonhomogeneous data were evaluated for significance using Wilcoxon's rank test [23].

RESULTS

Cannabinoid inhibition of adenylate cyclase activation in PMA plus ionomycin-stimulated lymphocytes. Activation of lymphocytes by concomitant treatment with PMA plus ionomycin produced a rapid but transient enhancement of cellular cAMP, which began at approximately 30 sec following cell activation, attained a maximum concentration at 3 min (4- to 5-fold increase), and returned to basal concentrations at approximately 10 min (Fig. 1). This increase in cellular cAMP following PMA plus ionomycin stimulation was inhibited completely by both Δ^9-THC (22 μM) and CP-55940 (5.2 μM), cannabinoid concentrations that have no effect on cell viability. The rationale for utilizing PMA plus ionomycin as an activation stimulus for these studies was that it allowed for an almost instantaneous and synchronized activation of the spleen cell preparation

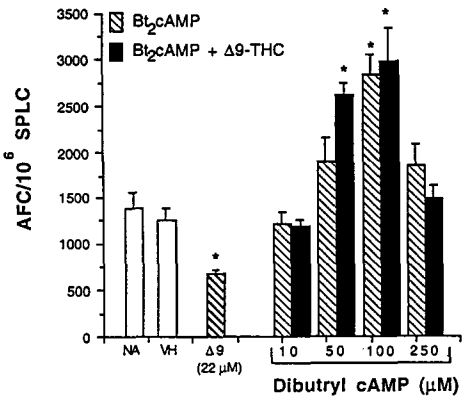

Fig. 3. Reversal of Δ^9-THC-mediated inhibition of the *in vitro* sRBC IgM AFC response by dibutyryl cAMP. Spleens from naive (NA) female B6C3F1 mice were isolated and made into a single cell suspension. Splenocytes were washed and adjusted to 1×10^7 cells/mL and transferred in 500-μL aliquots to the wells of a 48-well culture plate. Quadruplicate cultures were prepared with vehicle (VH; 0.01% DMSO) or Δ^9-THC (22 μM), then treated with dibutyryl cAMP (10–250 μM) and, last, sensitized with sRBC. Cultures were subsequently assayed using sRBC for their day 5 IgM antibody response by enumerating the number of antibody-forming cells (AFC). The bars represent the means ± SEM, as determined for each group, from one of four representative experiments. Key: (*)$P < 0.05$ as compared with the vehicle group (determined by Dunnett's *t*-test).

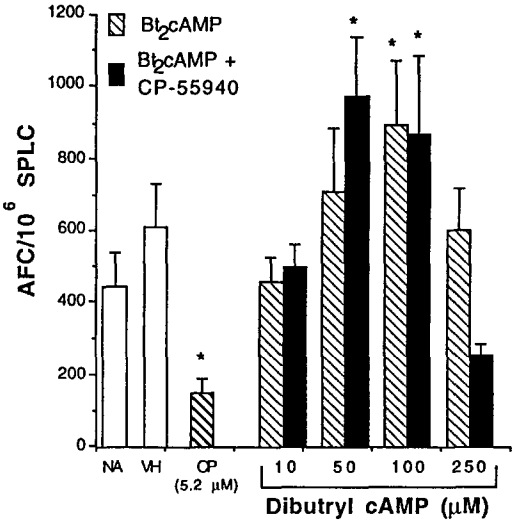

Fig. 4. Reversal of CP-55940-mediated inhibition of the *in vitro* sRBC IgM AFC response by dibutyryl cAMP. Spleens from naive (NA) female B6C3F1 mice were isolated and made into a single cell suspension. Splenocytes were washed and adjusted to 1×10^7 cells/mL and transferred in 500-μL aliquots to the wells of a 48-well culture plate. Quadruplicate cultures were prepared with vehicle (VH; 0.01% DMSO) or CP-55940 (5.2 μM), then treated with dibutyryl cAMP (10–250 μM) and, last, sensitized with sRBC. Cultures were subsequently assayed using sRBC for their day 5 IgM antibody response by enumerating the number of antibody-forming cells (AFC). The bars represent the means ± SEM, as determined for each group, from one of four representative experiments. Key: (*) $P < 0.05$ as compared with the vehicle group (determined by Dunnett's *t*-test).

and eliminated potential confounding effects by bypassing external receptors.

Effect of pertussis toxin pretreatment of spleen cells on cannabinoid-induced inhibition of cellular cAMP. Inhibition of adenylate cyclase by cannabinoid has been reported in a variety of cell preparations [10, 13, 24–26], including cannabinoid receptor-transfected CHO cells [4] and mouse spleen cells [16]. In cell membranes isolated from NG108-15 neuroblastoma X glioma hybrid cells and in N18TG2 neuroblastoma cells, this inhibition was found to be pertussis toxin sensitive, implicating the involvement of G_i [11]. *Bordetella pertussis* (pertussis toxin) acts by adding ADP-ribose to the α subunit of G_i. In this case, $G_{i\alpha}$ linked to ADP-ribose cannot inhibit adenylate cyclase [reviewed in Refs. 27 and 28]. As observed in neuroblastoma cells, inhibition of adenylate cyclase by cannabinoids in spleen cells was also reversed by a 24-hr pretreatment with pertussis toxin (Fig. 2). The most effective uncoupling of the cannabinoid receptor, as measured by adenylate cyclase activity, occurred at low pertussis toxin concentrations (0.1 ng/mL). An interesting aside to this series of experiments was the observation that 22 μM Δ^9-THC and 5.2 μM CP-55940 produced a comparable inhibition of adenylate cyclase activity. In previously reported findings, we observed that 22 μM Δ^9-THC and 5.2 μM CP-55940 also produce a comparable inhibition of the sRBC AFC response [15].

Reversal by cAMP of cannabinoid-mediated inhibition of the sRBC IgM AFC response and PMA plus ionomycin-induced splenocyte proliferation. The functional relevance of adenylate cyclase in lymphoid cells has been enigmatic. The studies described in Fig. 1 showed a rapid rise in cAMP shortly following lymphocyte activation, which was inhibited almost completely by Δ^9-THC (22 μM) and CP-55940 (5.2 μM) at concentrations that have been shown previously to inhibit a variety of immunological responses [15, 16, 29]. In an attempt to assess whether this cannabinoid-mediated inhibition of adenylate cyclase has functional relevance, studies were performed to determine whether inhibition by Δ^9-THC and CP-55940 of the sRBC AFC response and proliferative responses to PMA plus ionomycin could be reversed by experimentally increasing the cellular cAMP concentration using the membrane-permeable cAMP analog Bt_2cAMP. PMA plus ionomycin was used as an activation stimulus because it allowed for an almost instantaneous and synchronized activation of the spleen cell preparation. Concentrations of both Δ^9-THC (22 μM) and CP-55940 (5.2 μM), which were found previously to inhibit markedly both the IgM AFC response and lymphocyte proliferative responses [15, 16, 29], were utilized for these present studies. A relatively broad concentration response was performed with

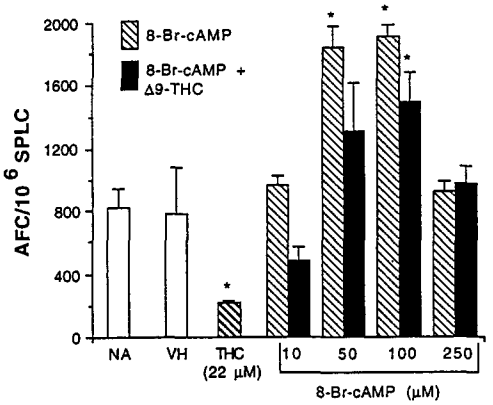

Fig. 5. Reversal of Δ^9-THC-mediated inhibition of the *in vitro* sRBC IgM AFC response by 8-bromo cAMP. Spleens from naive (NA) female B6C3F1 mice were isolated and made into a single cell suspension. Splenocytes were washed and adjusted to 1×10^7 cells/mL and transferred in 500-μL aliquots to the wells of a 48-well culture plate. Quadruplicate cultures were prepared with vehicle (VH; 0.01% DMSO) or Δ^9-THC (22 μM), then treated with 8-bromo cAMP (10–250 μM) and, last, sensitized with sRBC. Cultures were subsequently assayed using sRBC for their day 5 IgM antibody response by enumerating the number of antibody-forming cells (AFC). The bars represent the means ± SEM, as determined for each group, from one of four representative experiments. Key: (*) $P < 0.05$ as compared with the vehicle group (determined by Dunnett's *t*-test).

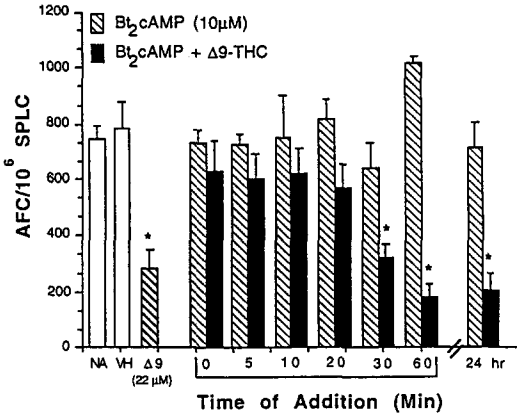

Fig. 6. Temporal addition of dibutyryl cAMP to Δ^9-THC-treated mouse spleen cells and its effect on the *in vitro* sRBC AFC response. Spleens from naive (NA) female B6C3F1 mice were isolated and made into a single cell suspension. Splenocytes were washed and adjusted to 1×10^7 cells/mL and transferred in 500-μL aliquots to the wells of a 48-well culture plate. Quadruplicate cultures were treated with vehicle (VH; 0.01% DMSO) or Δ^9-THC (22 μM) and sensitized with sRBC. At time 0–24 hr post-sensitization, the cells were dosed with dibutyryl cAMP (10 μM). Cultures were assayed using sRBC for their day 5 IgM antibody response by enumerating the number of antibody-forming cells (AFC). The bars represent the means ± SEM, as determined for each group, from one of three representative experiments. Key: (*) $P < 0.05$ as compared with the vehicle group (determined by Dunnett's *t*-test).

Bt_2cAMP ranging from 10 to 250 μM (final concentration in culture). Inhibition of the sRBC AFC response produced by both Δ^9-THC and CP-55940 was reversed through concomitant treatment with 10–100 μM Bt_2cAMP to the spleen cell cultures (Figs. 3 and 4). Additionally, as previously reported, Bt_2cAMP alone was found to enhance markedly the sRBC AFC response over this same range of concentrations. Bt_2cAMP concentrations equal to or greater than 250 μM were found to be inhibitory and above 500 μM to decrease cell viability markedly. To rule out the possibility that this reversal by Bt_2cAMP of the inhibitory effects produced by cannabinoids on the sRBC AFC response was not in some way either unique to this particular cAMP analog or perhaps being mediated through the release of butyrate, identical studies were performed with 8-Br-cAMP. Results from these studies revealed an almost identical profile of activity with membrane-permeable 8-Br-cAMP over the same concentration range as with Bt_2cAMP with a clear reversal of the Δ^9-THC-mediated inhibitory effects at 50 and 100 μM 8-Br-cAMP (Fig. 5).

A critical finding in the studies utilizing membrane-permeable cAMP analogs was the observation that temporal addition of Bt_2cAMP was only capable of reversing the inhibition of the AFC response by Δ^9-THC if added to culture within the first 20–30 min following antigen sensitization and was ineffective at times 30 min post-antigen sensitization (Fig. 6). The significance of this observation is that, although the functional response (i.e. AFC response) was measured 5 days following cell treatment, the role played by cAMP occurred during the first 30 min after addition of antigen. Neither 22 μM Δ^9-THC nor 5.2 μM CP-55940 was found to decrease cell viability in cultured splenocytes. Similarly, direct addition of Bt_2cAMP (1–100 μM) to spleen cell cultures demonstrated reversal by both Δ^9-THC and CP-55940 of the inhibition of PMA plus ionomycin-induced lymphocyte proliferative response (Fig. 7).

Reversal by pertussis toxin of cannabinoid-mediated inhibition of the sRBC IgM AFC response. To explore further the functional significance of adenylate cyclase inhibition by cannabinoids in spleen cells and to determine whether the concomitant decrease in immune competence occurs through a G_i-protein coupled mechanism, spleen cells were pretreated for 24 hr in the presence of pertussis toxin (0.1 to 100 ng/mL) prior to treatment with either Δ^9-THC or CP-55940 and antigen. As with Bt_2cAMP, pertussis toxin abrogated the inhibitory effects produced by cannabinoids on the sRBC AFC response (Figs. 8 and 9). Short preincubations (either 1 or 4 hr) of spleen cells with pertussis toxin were also tested; however, these were found to be insufficient to reverse cannabinoid inhibition of the sRBC AFC response (data not shown).

DISCUSSION

The cannabinoid receptor CB1 is a novel G-

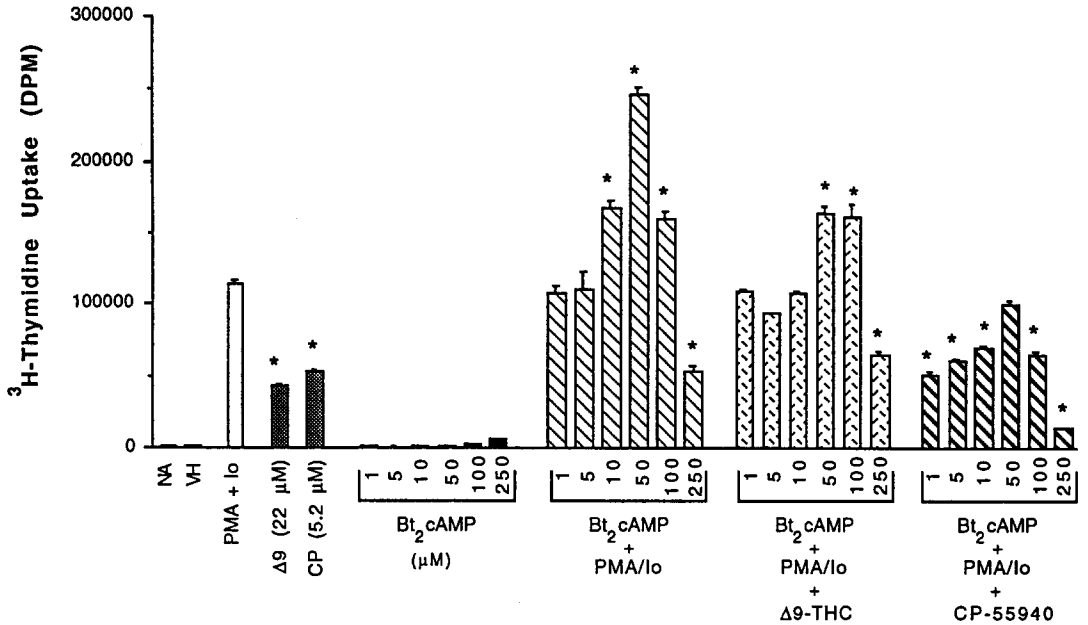

Fig. 7. Reversal of Δ^9-THC- and CP-55940-mediated inhibition of PMA plus ionomycin-induced proliferation by dibutyryl cAMP. Spleens from naive (NA) female B6C3F1 mice were isolated and made into a single cell suspension. Splenocytes were washed and adjusted to 5×10^6 cells/mL and transferred in 200-μL aliquots to the wells of a 96-well culture plate. Quadruplicate cultures were treated with either vehicle (VH; 0.01% DMSO), Δ^9-THC (22 μM) or CP-55940 (5.2 μM). After preincubating for 10 min at 25°, the cells were treated sequentially with dibutyryl cAMP and PMA + ionomycin. Cultures were incubated for 48 hr at 37° and then pulsed for an additional 24 hr with [^{3}H]thymidine. Cells were harvested using a PHD cell harvester, and the data are expressed as the amount of [^{3}H]thymidine uptake (dpm). The bars represent the means ± SEM, as determined for each group, from one of five representative experiments. Key: (*) $P < 0.05$ as compared with the vehicle group (determined by Dunnett's t-test).

protein coupled receptor recently identified in both rat [4] and human brain [30], human testis [6], mouse spleen [15] human lymphoid cell lines and primary lymphoid cells [17]. This receptor appears to be most abundant in select brain regions, primarily cortex, cerebellum and striatum [5]. DNA sequencing analyses, which show greater than 97% receptor homology between those expressed in human and rat brain, suggest that CB1 is highly conserved between species. Although DNA sequencing has not yet been performed for CB1 in mouse spleen cells, results from radioligand binding analysis utilizing the synthetic bicyclic radioligand [^{3}H]CP-55940 are strikingly similar to those previously reported for neuronal tissue in other animal species where CB1 has been sequenced: single site binding possessing a K_d of approximately 910 pM [15]. Previously reported binding analysis using various neuronal tissue preparations indicates single site binding with a K_d in the 1 nM range [5, 8]. Further evidence supporting the expression of CB1 on mouse spleen cells was the identification of CB1 mRNA transcripts by RNA PCR and RNase protection [15]. Recently, Munro and coworkers [18] reported the identification of a cannabinoid receptor subtype, CB2, in the germinal center of human spleen, which was not detectable in brain tissue preparations. The relevance/function of both CB1 and CB2 is presently enigmatic. However, recently, arachidonylethanolamide, an arachidonic acid isolated from porcine brain, was reported to inhibit the specific binding of a cannabinoid-radiolabeled probe to synaptosomal membranes in a manner typical of competitive ligands [31], suggesting that select eicosinoids may be endogenous ligands for cannabinoid receptors.

Signal transduction through CB1 and presumably CB2 is mediated through inhibition of the adenylate cyclase/cAMP second messenger pathway [9–13, 16]. The present studies suggest that cannabinoid-mediated inhibition of the AFC response and proliferative response to PMA plus ionomycin by spleen cells occurs through the inhibition of adenylate cyclase, which may be a critical event in the early stages of lymphocyte activation. This is supported by the observation that, under normal conditions, spleen cells activated by phorbol ester plus the calcium ionophore ionomycin, exhibited a very rapid as well as transient increase in intracellular cAMP. This increase in cellular cAMP was first detected between 30 and 60 sec following cell activation, reaching a maximum at 3 min which returned back to basal levels within approximately 10 min. This activation of adenylate cyclase is mediated indirectly through the activation of protein kinase C (PKC) and is not a direct effect by PMA plus ionomycin on adenylate cyclase. This conclusion is based on

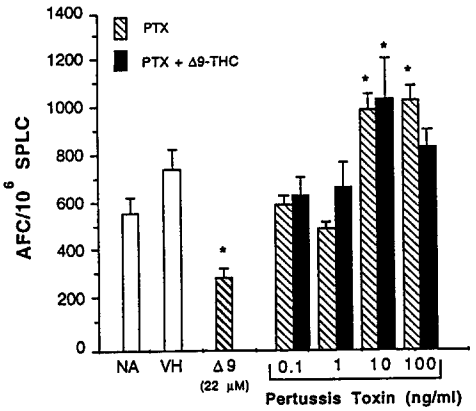

Fig. 8. Reversal of Δ^9-THC-mediated inhibition of the *in vitro* sRBC IgM AFC response by pertussis toxin. Spleens from naive (NA) female B6C3F1 mice were isolated and made into a single cell suspension. Splenocytes were washed and adjusted to 1×10^7 cells/mL and aliquoted into 15-mL polypropylene tubes. Both naive and vehicle groups were left untreated for 18 hr at 37°, while the treatment group was incubated with pertussis toxin (0.1 to 100 ng/mL) for 18 hr at 37°. Cells were transferred in 500-μL aliquots to the wells of a 48-well culture plate, and quadruplicate cultures were prepared with vehicle (VH; 0.01% DMSO) or Δ^9-THC (22 μM) and sensitized with sRBC. Cultures were subsequently assayed using sRBC for their day 5 IgM antibody response by enumerating the number of antibody-forming cells (AFC). The bars represent the means ± SEM, as determined for each group, from one of three representative experiments. Key: (*) $P < 0.05$ as compared with the vehicle group (determined by Dunnett's *t*-test).

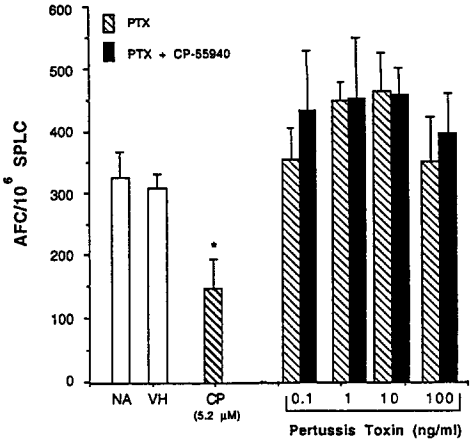

Fig. 9. Reversal of CP-55940-mediated inhibition of the *in vitro* sRBC IgM AFC response by pertussis toxin. Spleens from naive (NA) female B6C3F1 mice were isolated and made into a single cell suspension. Splenocytes were washed and adjusted to 1×10^7 cells/mL and aliquoted into 15-mL polypropylene tubes. Both naive and vehicle groups were left untreated for 18 hr at 37°, while the treatment group was incubated with pertussis toxin (0.1 to 100 ng/mL) for 18 hr at 37°. Cells were transferred in 500-μL aliquots to the wells of a 48-well culture plate, and quadruplicate cultures were prepared with vehicle (VH; 0.01% DMSO) or CP-55940 (5.2 μM) and sensitized with sRBC. Cultures were subsequently assayed using sRBC for their day 5 IgM antibody response by enumerating the number of antibody-forming cells (AFC). The bars represent the means ± SEM, as determined for each group, from one of three representative experiments. Key: (*) $P < 0.05$ as compared with the vehicle group (determined by Dunnett's *t*-test).

the fact that the PKC inhibitor chelerythrine abrogated the activation of PKC by PMA–ionomycin treatment (unpublished observation). Similar bursts in cellular cAMP following mitogenic stimulation of lymphocytes have been reported by others [32–34]. Conversely, spleen cells activated with PMA plus ionomycin in the presence of cannabinoids do not exhibit this rise in cellular cAMP due to the inhibition of adenylate cyclase. This has also been demonstrated in Δ^9-THC-treated splenocytes following activation with the diterpene forskolin [16]. These findings suggest that a rapid increase in cellular cAMP may be a critical signal transduction event during lymphocyte activation.

Establishing a direct link between rapidly occurring biochemical changes associated with signal transduction events and functional changes, which in many cases require several days such as cytokine secretion, proliferation or the production of antibody, is extremely difficult. The findings from this series of studies represent the first step in establishing such a link between the inhibition of adenylate cyclase in spleen cells by cannabinoids and a loss of immune function. It is important to emphasize that, although by no means confirmatory, several critical observations from these studies support this premise. Biochemically, the present findings clearly establish that cannabinoids almost completely abrogated the rapid rise in intracellular cAMP which follows activation of splenocytes with phorbol ester and calcium ionophore. Similar results were obtained following forskolin stimulation, a diterpene which directly activates the enzyme, and is therefore indicative that cannabinoids inhibit activation of adenylate cyclase. Equally important is the fact that pretreatment of spleen cells with pertussis toxin reversed the inhibitory effects produced by cannabinoids on adenylate cyclase activity. This pertussis toxin sensitivity is not unique to splenocytes and has been reported by others in other experimental model systems in characterizing the cannabinoid receptor [4, 11, 35]. Taken together, these results suggest that cannabinoids inhibit the intracellular rise of cAMP during lymphocyte activation through the inhibition of adenylate cyclase by a pertussis toxin-sensitive mechanism, most likely involving G_i.

In an attempt to correlate the functional significance of these biochemical changes, an initial and basic premise tested was that if inhibition of cellular cAMP was, in fact, critical to spleen cell function, immune inhibition by cannabinoids should be reversed in the presence of Bt_2cAMP or 8-Br-cAMP, membrane-permeable cAMP analogs. Interestingly, cannabinoid inhibition of both the spleen cell proliferative responses to PMA plus

ionomycin as well as the sRBC antibody response was, in fact, reversed by low concentrations of cAMP analogs (10–50 μM). Additionally, temporal addition studies revealed that the reversal of the sRBC response by exogenous cAMP was restricted to the first 30 min following antigen sensitization. It is important to emphasize that although PMA plus ionomycin treatment of spleen cells clearly induced a very rapid increase in cellular cAMP within 3 min, this activation signal is virtually instantaneous, bypassing cell surface receptors and activating all spleen cells simultaneously. In contrast, lymphocyte activation in response to antigen sensitization by sRBC is slower and dependent on antigen processing and presentation by antigen presenting cells prior to lymphocyte activation. Under this second condition, lymphocyte activation obviously would not be synchronized such that all cells would not be activated in unison instantaneously following sRBC sensitization. With this in mind, although the kinetics of the sRBC AFC response reversal by Bt_2cAMP are slightly different than what would be expected for reversal of the PMA plus ionomycin proliferative response, nonetheless, the findings are in agreement and further support the premise that inhibition of adenylate cyclase by cannabinoids affects an early cAMP-dependent lymphocyte activation event. The temporal addition studies are especially significant in that although they measure a response requiring 5 days, nonetheless they clearly indicate that cAMP is critical to events occurring during the first 30 min following antigen sensitization. Further evidence supporting the role of adenylate cyclase inhibition in mediating at least some aspects of immunosuppression by cannabinoids was the finding that as with forskolin-stimulated cellular cAMP accumulation, pertussis toxin reversed the inhibitory influences associated with either Δ^9-THC or CP-55940 on the sRBC AFC response.

Although the present studies strongly suggest that the inhibition of certain immune responses by Δ^9-THC and structurally related cannabinoids is mediated through inhibition of adenylate cyclase activity, which results in a depletion of the cytosolic cAMP pool in splenocytes, there currently exist two opposing views ascribed to the functional role of the adenylate cyclase/cAMP second messenger system with respect to lymphocytes. One view supports the premise that cAMP is an early but transient signal associated with lymphocyte activation. This is consistent with numerous studies including our own, which clearly demonstrate that early during lymphocyte activation there occurs a modest 2- to 5-fold transient enhancement of cytosolic cAMP that can persist up to approximately 30 min after the initial activating signal [32–34]. This enhancement in cellular cAMP has been shown by others to be accompanied by corresponding protein kinase A (PKA)-associated phosphorylation events in which the greatest magnitude of PKA-mediated phosphorylation also occurs during the first 30 min following lymphocyte activation [36]. Related to this observation has been the finding that low concentrations of Bt_2cAMP (0.1 to 10 μM) induce the same degree of lymphocyte RNA synthesis as that induced by the T-cell lectin phytohemagglutinin [36]. Conversely, Bt_2cAMP at concentrations in excess of 100 μM markedly inhibit RNA synthesis. A second but opposing view is that cAMP is a strong inhibitory signal in lymphocytes. This is supported by studies in which membrane-permeable cAMP analogs added directly to activated lymphocyte cultures were found to inhibit markedly cell cycle progression. Both B- and T-cells have been shown to be arrested in transition from G_1 to S phase following experimental elevation of cytosolic cAMP: in T-cells by high concentrations of cAMP analogs, 250–500 μM [37], or in B-cells following stimulation with the diterpene adenylate cyclase activator, forskolin [38]. It is important to emphasize that, on closer examination of the results, the findings are not contradictory but describe different modulatory effects for cAMP at different times during cell cycle progression and at different cyclic nucleotide concentrations. Without exception, even those studies that support the role of cAMP as being an important intracellular mediator of lymphocyte activation have demonstrated clearly that high nonphysiologically relevant concentrations of cAMP (in excess of 100 μM) produce marked inhibition of various cellular processes, and can be mimicked *in vitro* with membrane-permeable cAMP analogs [36].

Much confusion still exists pertaining to the role of the adenylate cyclase/cAMP signal transduction pathway in lymphocyte activation and function. Nonetheless, the present results clearly demonstrate that inhibition of lymphocyte proliferation or the IgM sRBC AFC response by both Δ^9-THC and CP-55940 is accompanied by a marked inhibition of adenylate cyclase activity in spleen cells. This inhibition can be abrogated effectively by restoring cytosolic cAMP with micromolar concentrations of membrane-permeable cAMP analogs or in the case of the sRBC response, through the inactivation of $G_{i\alpha}$ by pertussis toxin. These findings, together with the kinetics of splenocyte inhibition by cannabinoids and the kinetics associated with the reversal of inhibition by exogenous cAMP, strongly support the role of a G_i-protein coupled cannabinoid receptor that, upon ligand-receptor binding, inhibits adenylate cyclase activity which, in turn, blocks the conversion of ATP to cAMP, an obligatory signal transduction event during lymphocyte activation.

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