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Inhibition of Protein Kinase A and Cyclic AMP Response Element (CRE)-Specific Transcription Factor Binding by Δ^9-Tetrahydrocannabinol (Δ^9-THC)

A PUTATIVE MECHANISM OF CANNABINOID-INDUCED IMMUNE MODULATION

*Woo S. Koh, Robert B. Crawford and Norbert E. Kaminski**

DEPARTMENT OF PHARMACOLOGY AND TOXICOLOGY AND DEPARTMENT OF PATHOLOGY, MICHIGAN STATE UNIVERSITY, EAST LANSING, MI 48824 U.S.A.

ABSTRACT. Δ^9-Tetrahydrocannabinol (Δ^9-THC) binding to cannabinoid receptors induces an inhibition in adenylate cyclase activity through the engagement of a pertussis toxin-sensitive GTP-binding protein. In this study we investigated the ramifications of decreased cyclic AMP (cAMP) formation by Δ^9-THC on signaling events through the cAMP pathway distal to adenylate cyclase in mouse splenocytes. Δ^9-THC treatment produced a marked and concentration-related decrease in forskolin-inducible protein kinase A (PKA) activity. This decrease in kinase activity was due to an inhibition in cAMP formation and not through a direct effect on the kinase as evidenced by the fact that PKA activity could not be modulated directly by Δ^9-THC in the presence of exogenous cAMP. One of the primary roles of PKA in this signaling pathway is to activate transcription factors for subsequent binding to cAMP response elements (CRE) present in the promoter region of cAMP-responsive genes. In the present studies, we observed that forskolin treatment of splenocytes resulted in a rapid activation of *trans*-acting factor binding to the CRE, which peaked at 30–60 min and whose binding was repressed concentration dependently in the presence of Δ^9-THC. As with forskolin, mitogenic stimulation including anti-CD3 mAb or phorbol ester plus ionomycin treatment of splenocytes induced CRE binding activity, which was maximal around 60 min and was suppressed by Δ^9-THC treatment. In conclusion, these data indicate that cAMP-mediated signal transduction is inhibited by Δ^9-THC and consequently leads to a decrease in the activation of transcription factors that bind to CRE regulatory sites. BIOCHEM PHARMACOL 53;10: 1477–1484, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. cannabinoids; CRE; delta-9-tetrahydrocannabinol; immune suppression; protein kinase A; cAMP cascade

Δ^9-THC,† the primary psychoactive constituent of marihuana [1], is also widely established as being immunosuppressive [2–6]. The mechanism responsible for biological activity by Δ^9-THC and related cannabinoid compounds, although poorly understood, is now known to be mediated, at least in part, through cannabinoid receptors. Thus far, two major types of cannabinoid receptors have been isolated and cloned: CB1, which is predominantly expressed within the CNS [7], and CB2, the receptor-type primarily expressed within the immune system [8]. One of the major signaling pathways implicated in the biological actions exerted following ligand binding to cannabinoid receptors has been the cAMP cascade. Cannabinoid compounds induce an inhibition of adenylate cyclase activity through a pertussis-toxin-sensitive inhibitory GTP-binding protein in neuronal and immune cells that leads to decreased intracellular formation and accumulation of cAMP [5, 9, 10]. The relevance of this action to immune inhibition by cannabinoids likely pertains to the inhibition of the cAMP "burst" that occurs in lymphoid cells within several minutes following their activation [5, 11–15] and is then rapidly followed by activation of PKA, a cAMP-dependent kinase [16]. Although cAMP has historically been viewed by some as a negative regulator of lymphocyte function, the modest increase observed shortly after cell activation is now generally considered to be an essential factor in immune responses [17, 18]. This increase in the intracellular cAMP

* Corresponding author: Dr. Norbert E. Kaminski, Department of Pharmacology and Toxicology, B-330 Life Sciences Bldg., Michigan State University, East Lansing, MI 48824. Tel. (517) 353-3786; FAX (517) 353-8915.

† *Abbreviations*: Δ^9-THC, delta-9-tetrahydrocannabinol; PMA, phorbol-12-myristate-13-acetate; CB1, cannabinoid receptor type 1; CB2, cannabinoid receptor type 2; cAMP, cyclic adenosine 3′:5′-monophosphate; CRE, cAMP response element; anti-CD3, monoclonal antibody against the T-cell receptor/CD3 complex; PKA, protein kinase A; PKI, protein kinase A inhibitor; IL-1, interleukin-1; IL-2, interleukin-2; CREB, cAMP response element binding protein; ATF, activating transcription factor; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay(s); and EBSS, Earle's balanced salt solution.

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concentrations results in the occupation of the cAMP-binding sites on the regulatory subunit of the PKA holoenzyme complex to induce the dissociation of the regulatory and catalytic subunits. The free catalytic subunits are then active and believed to mediate phosphorylation of cytoplasmic, membrane, and nuclear substrates [19]. The activation of PKA by cAMP subsequently induces the transcription of a variety of genes via the binding of homo- and heterodimers of CREB and ATF to a DNA binding motif called the CRE [20, 21] present in the promoter region of cAMP-regulated genes.

The role of cannabinoid receptor–ligand interactions in mediating changes in cell signaling via the cAMP pathway, and their impact on alteration of normal physiologic processes continue to remain obscure. Recently, Δ^9-THC and the structurally related cannabinoid, cannabinol, were shown to inhibit the cAMP signaling cascade at a number of checkpoints along this pathway including adenylate cylase, PKA, and the terminal step of this signal pathway, activation and binding of CRE specific *trans*-acting factors in the murine T-cell line EL4.IL-2 [22]. In light of this, the objective of the present studies was to determine whether inhibition of adenylate cyclase by Δ^9-THC would similarly lead to a decrease in PKA activity and subsequently to an inhibition of nuclear factor binding to CREs in primary murine leukocytes. Toward this end, modulation of CRE binding activity in mouse splenocytes was examined following a variety of stimuli including anti-CD3 mAb, PMA plus ionomycin, and forskolin in the presence and absence of Δ^9-THC.

MATERIALS AND METHODS

Animal

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 sawdust bedding (4 mice or 2 rats 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.

Analysis of PKA Activity

PKA assay was performed as previously described [16] with modifications [22]. In brief, splenocytes were isolated and washed once with phosphate-buffered saline (pH 7.2), followed by lysis of red blood cells with Gey's solution. Cells were washed in EBSS and lysed in ice-cold lysis buffer [0.25 M sucrose, 50 mM Tris–HCl (pH 7.5), 5 mM EGTA, 1 mM PMSF, 0.1 mM DTT, 0.1% Triton X-100, and 10 μg/mL each of leupeptin and aprotinin] at 4° by gentle sonication in order to maintain the functional integrity of the cannabinoid receptor as it is anchored in the membrane (i.e. twice at 60 Hz for 5 sec). Lysates were centrifuged at 270 *g* for 2 min, and aliquots of the cell extract (supernatant) were incubated with appropriate concentrations of Δ^9-THC for 5 min in triplicate for use in the PKA assay (Gibco BRL, Grand Island, NY). The reaction mixture of 40 μL contained 10 μL of cell extract, 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 100 μM ATP (20 μCi/mL [γ-[^{32}P]ATP), 0.25 mg/mL bovine serum albumin, and 50 μM Kemptide. Appropriate samples were treated with 50 μM forskolin. The background level of each group was measured in the presence of 1 μM PKI(6-22)amide, and total activity was measured in the presence of exogenous cAMP (10 μM). Samples were incubated at 37° for 10 min. Phosphocellulose discs were then spotted with 20 μL of sample followed by two acid washes [1% (v/v) phosphoric acid] and one water wash. The amount of ^{32}P was quantified by scintillation counting.

Preparation of Nuclear Extracts

Nuclear extracts were prepared as previously described [22, 23]. Splenocytes were treated with either vehicle (0.1% ethanol) or one of the following stimuli: 50 μM forskolin, 2 μg/mL anti-CD3, or 80 nM PMA plus 1 μM ionomycin, in the presence or absence of 22 μM Δ^9-THC. Treated and untreated splenocytes were isolated, lysed by incubation for 15 min in a hypotonic buffer (pH 7.5, 10 mM HEPES, and 1.5 mM $MgCl_2$), and pelleted by centrifugation at 6700 *g* for 5 min. The nuclei were extracted in hypertonic buffer (30 mM HEPES, 1.5 mM $MgCl_2$, 450 mM NaCl, 0.3 mM EDTA, 10% glycerol), supplemented with 1 mM DTT, 1 mM PMSF, and 1 μg/mL of aprotinin and leupeptin. After centrifugation (15,000 *g*, 15 min), the supernatant was diluted with 2 vol. of buffer without NaCl, rapidly frozen, and stored at −70°.

EMSA

EMSA were performed as previously described [22]. Briefly, two double-stranded deoxyoligonucleotides containing the CRE motif (5′-TGACGTCA-3′) [24] were synthesized in the Biotechnology Facility of Michigan State University, annealed, and end-labeled with ^{32}P. Nuclear extracts (3 μg) were incubated with ^{32}P-labeled probe in binding buffer [30 mM HEPES, 0.5 μg of poly(dI-dC), 100 mM NaCl, 1.5 mM $MgCl_2$, 0.3 mM EDTA, 10% glycerol, 1 mM DTT, 1 mM PMSF, and 1 μg/mL of aprotinin and leupeptin] for 10 min on ice. Reaction products were resolved by electrophoresis in native 5% polyacrylamide gels in 1× TBE buffer (89 mM Tris-Cl, 89 mM boric acid, and 2 mM EDTA). After electrophoresis, gels were dried and subjected to autoradiography.

Statistical Analysis of Data

The mean ± SEM was determined for each treatment group of a given experiment. Homogeneous data were evaluated by a parametric analysis of variance, and Dun-

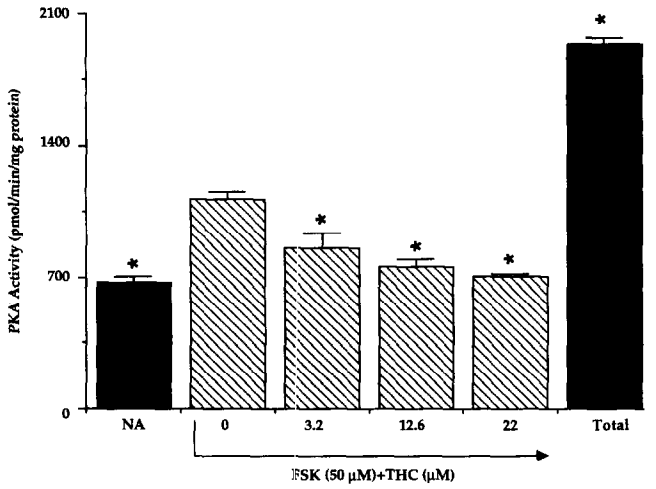

FIG. 1. Concentration-dependent inhibition by Δ^9-THC of forskolin-inducible PKA activity. Isolated splenocytes were lysed in ice-cold lysis buffer at 4° by sonication twice at 60 Hz for 5 sec. Cellular debris was removed by centrifugation for 2 min in a microcentrifuge. Cell extracts were preincubated with Δ^9-THC or 0.1% EtOH 10 min prior to reaction and then incubated in the presence or absence of forskolin (50 μM). The background level of each group was measured in the presence of 1 μM PKI(6-22)amide. NA = naive. Total PKA activity in the cell lysate was determined following the direct addition of exogenous cAMP (10 μM). Then phosphocellulose discs were spotted with 20 μL of sample and washed two times with 1% (v/v) phosphoric acid and then once with water. The amount of ^{32}P was quantified by scintillation counting. Values from one of three independent experiments are expressed as the means ± SEM for triplicate samples as determined for each group. Key: (*)$P < 0.05$, compared with the forskolin control group (determined by Dunnett's *t*-test).

nett's two-tailed *t*-test [25] was used to compare treatment groups with the control when significant differences were observed.

RESULTS

Inhibition of Forskolin-Induced PKA Activity by Δ^9-THC

To investigate the effect of Δ^9-THC on cAMP-inducible signal transduction, PKA activity was assessed using the PKA specific synthetic peptide Kemptide. Experiments were designed to quantify PKA activity following the formation of endogenous cAMP by forskolin stimulation of membrane-associated adenylate cyclase. The integrity of adenylate cyclase in this preparation was confirmed by the fact that forskolin treatment (50 μM) induced a rapid enhancement of PKA activity. Moreover, treatment of the cell extracts containing membrane fragments with Δ^9-THC, 5 min before forskolin stimulation, blocked the induction of kinase activity in a concentration-dependent manner as is shown in Fig. 1. PKA activity was 165.3 ± 5.6, 127.7 ± 11.2, 112.8 ± 5.6, and 105.5 ± 0.9% of the naive control in the presence of Δ^9-THC at 0, 3.2, 12.6, and 22 μM, respectively. At the highest Δ^9-THC concentration (22 μM), PKA activity was almost decreased back to basal levels, suggesting that modulation of this kinase can be readily achieved by cannabinoids. Total PKA activity in the preparations was determined by direct addition of exogenous cAMP to the cell lysates in the absence of Δ^9-THC. It is important to emphasize that the decrease in PKA activity was not due to a direct inhibition of the kinase by Δ^9-THC but rather to a decrease in cAMP formation. This is demonstrated by the studies shown in Fig. 2, in which basal or total PKA activity, as measured in the presence or absence of exogenous cAMP (10 μM), respectively, could not be altered by Δ^9-THC.

Inhibition by Δ^9-THC of Forskolin-Induced CRE Binding

To assess whether the inhibition of PKA by Δ^9-THC in turn attenuates the terminal step within this signaling cascade, that being the binding of *trans*-activating factors to CRE DNA motifs, nuclear extracts from forskolin (50 μM)-stimulated splenocytes were prepared for EMSA at 0, 15, 30, 60, 90, or 120 min in the presence or absence of Δ^9-THC (Fig. 3). Binding activity to the CRE motif as assayed in nuclear extracts from spleen cells was time dependent. These gel shift assays also revealed that binding to the CRE motif was rapidly but transiently up-regulated, with maximum binding detected at 30–60 min following

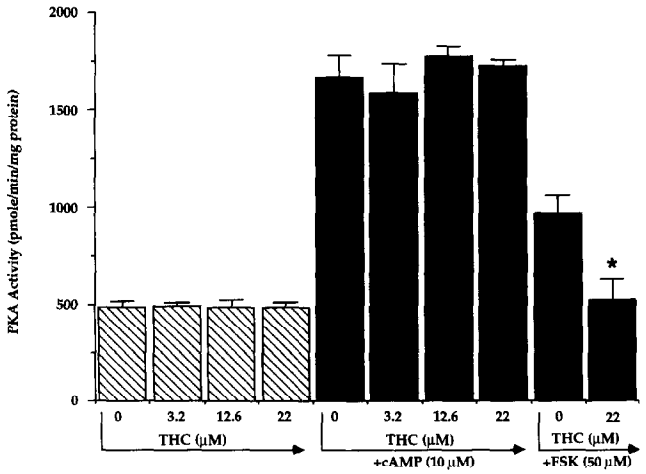

FIG. 2. Effects of Δ^9-THC on basal or total PKA activities. Isolated splenocytes were lysed in ice-cold lysis buffer at 4° by sonication twice at 60 Hz for 5 sec. Cellular debris was removed by centrifugation for 2 min in a microcentrifuge. Cell extracts were preincubated with Δ^9-THC or 0.1% EtOH 10 min prior to reaction and then incubated in the presence or absence of forskolin (50 μM). The background level of each group was measured in the presence of 1 μM PKI(6-22)amide. Total PKA activity in the cell lysate was determined following the direct addition of exogenous cAMP (10 μM). Then phosphocellulose discs were spotted with 20 μL of sample and washed two times with 1% (v/v) phosphoric acid and then once with water. The amount of ^{32}P was quantified by scintillation counting. Values from one of three independent experiments are expressed as the means ± SEM for triplicate samples as determined for each group. Key: (*)$P < 0.05$, compared with the forskolin control group (determined by Dunnett's *t*-test).

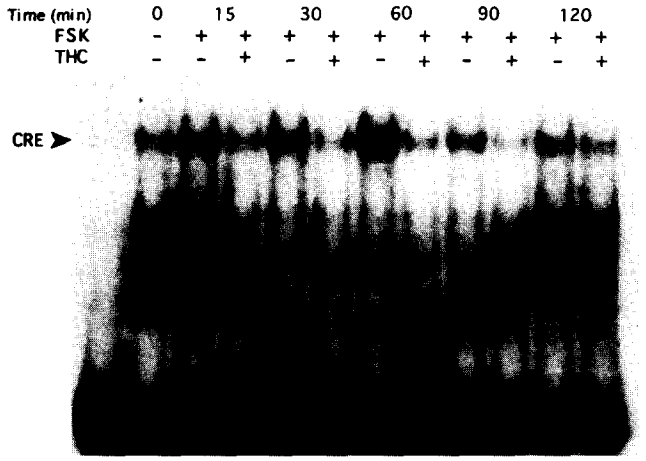

FIG. 3. Time-dependent inhibition of forskolin-induced CRE binding by Δ^9-THC in mouse splenocytes as demonstrated by EMSA. Nuclear extract (3 μg) from forskolin (50 μM)-stimulated splenocytes in the presence or absence of Δ^9-THC (22 μM) was incubated with 0.5 μg of poly(dI-dC), 100 mM NaCl, and ^{32}P-labeled DNA probe (30,000 cpm) in binding buffer on ice for 10 min followed by electrophoretic separation on a 5% polyacrylamide gel. The result represents one of five independent experiments.

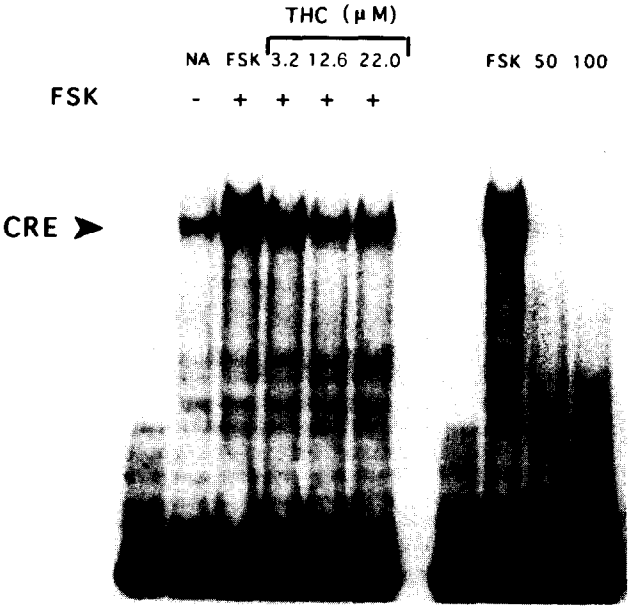

FIG. 4. Concentration-dependent inhibition by Δ^9-THC of forskolin-inducible CRE-binding activity as demonstrated by EMSA. Nuclear extracts were prepared from the splenocytes stimulated with forskolin (50 μM) for 60 min in the presence of various concentrations (0, 3.2, 12.6, 22 μM) of Δ^9-THC. Three micrograms of nuclear extract was incubated with 0.5 μg of poly(dI-dC), 100 mM NaCl, and ^{32}P-labeled DNA probe (30,000 cpm) in binding buffer on ice for 10 min followed by electrophoretic separation on a 5% polyacrylamide gel. Unlabeled CRE probe (50 or 100 ng) was added to compete with ^{32}P-labeled DNA probe (30,000 cpm) in the binding with nuclear protein from forskolin (50 μM)-stimulated splenocytes. The result represents one of five independent experiments.

forskolin stimulation. This forskolin-induced increase in CRE binding was found to gradually begin declining, as first apparent at approximately 90 min, and returned back to basal levels by 2 hr. Interestingly, one major band was observed at all of the time points assayed. A second minor band formed by a larger binding complex (i.e. above the major binding complex), although not readily visible in Fig. 3 but apparent in Fig. 4, was observed routinely at 60 min following forskolin stimulation. Δ^9-THC treatment of splenocytes prior to forskolin stimulation almost completely abolished the enhancement of CRE binding at every time point tested with the upper minor band being inhibited completely at all of the concentrations tested (Fig. 4). Furthermore, the inhibition of the major CRE binding complex by Δ^9-THC treatment appeared to be concentration related (Fig. 4). Quantitation of the band intensities by densitometry for the major CRE binding complex revealed a 43, 78, and 70% inhibition of binding as compared with the forskolin control in the presence of Δ^9-THC at 3.2, 12.6, and 22 μM, respectively. Moreover, the forskolin-induced CRE binding was specific as demonstrated by the ability of ^{32}P-unlabeled CRE to effectively inhibit protein binding to the radiolabeled CRE probe (Fig. 4).

Inhibition of Anti-CD3 mAb or PMA plus Ionomycin Induced CRE Binding Activity by Δ^9-THC

While the results shown above indicate that Δ^9-THC inhibits the activation and binding of CRE-specific *trans*-acting factors that are induced by forskolin, the involvement of these factors is not clear during mitogenic activation of spleen cells. Therefore, we assayed for the presence of activated CRE binding proteins in nuclear extracts prepared from spleen cells at various times following stimulation with either PMA plus ionomycin or the T-cell specific activator anti-CD3. As observed with forskolin, both PMA plus ionomycin and anti-CD3 induced an increase in the binding of CRE specific proteins (Figs. 5 and 6). The kinetics of activation following either of the mitogenic stimuli was similar to that observed with forskolin, with maximal binding being observed around 60 min. One difference in the banding pattern produced between the two different stimuli was that PMA plus ionomycin treatment, which activates all of the leukocytes in this mixed cell preparation, induced only one major binding complex, whereas anti-CD3 treatment, which only activates T-cells, induced the major binding complex (identified by the arrow) as well as the second minor binding complex (above the major complex) previously observed with forskolin treatment at 60 min post-stimulation (Fig. 4). Moreover, this increase in binding activity at 60 min following anti-CD3 or PMA plus ionomycin stimulation was also inhibited by Δ^9-THC (22 μM) treatment with the binding activity of the minor complex in the anti-CD3-stimulated cells being abolished completely in the presence of the cannabinoid. As above, the specificity of CRE binding activity was confirmed by competition with unlabeled CRE.

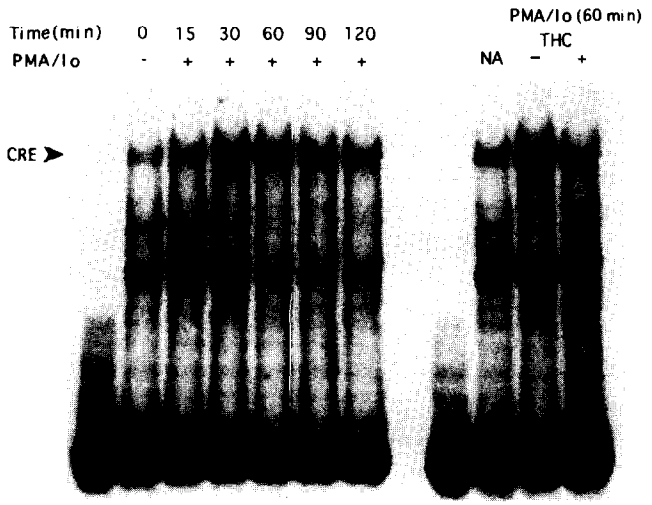

FIG. 5. Inhibition by Δ^9-THC of PMA plus ionomycin-induced CRE-binding activity as demonstrated by EMSA. Nuclear extracts (3 μg) from forskolin (50 μM) or PMA (80 nM) plus ionomycin (1 μM)-stimulated splenocytes in the presence or absence of Δ^9-THC (22 μM) were incubated with 0.5 μg of poly(dI-dC), 100 mM NaCl, and ^{32}P-labeled DNA probe (30,000 cpm) in binding buffer on ice for 10 min followed by electrophoretic separation on a 5% polyacrylamide gel. The result represents one of three independent experiments.

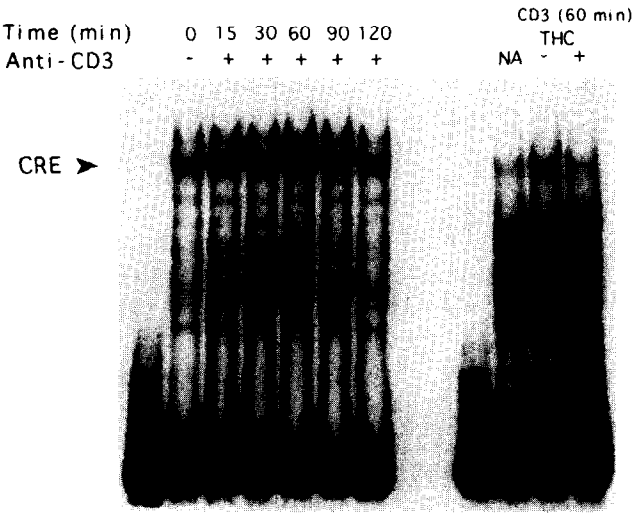

FIG. 6. Inhibition by Δ^9-THC of anti-CD3-induced CRE-binding activity as demonstrated by EMSA. Nuclear extracts (3 μg) from forskolin (50 μM) or anti-CD3 mAb-stimulated splenocytes in the presence or absence of Δ^9-THC (22 μM) were incubated with 0.5 μg of poly(dI-dC), 100 mM NaCl, and ^{32}P-labeled DNA probe (30,000 cpm) in binding buffer on ice for 10 min followed by electrophoretic separation on a 5% polyacrylamide gel. NA = naive. The result represents one of three independent experiments.

DISCUSSION

In the present studies, we demonstrated that treatment of mouse splenocytes with Δ^9-THC, which rapidly induces an inhibition of adenylate cyclase activity through binding to cannabinoid receptors [5, 7, 10, 26, 27], results in the attenuation of PKA activity and a decrease in binding of *trans*-activating factors to CRE. We believe that this negative regulation of the cAMP signaling cascade by cannabinoid compounds represents a critical component of the mechanism by which this class of compounds mediate their immunoinhibitory activity at the signal transducing level. Although much confusion still exists regarding the role of the cAMP signaling cascade in immune regulation, it is now evident from an increasing number of studies, including those presented here, that this pathway is actively engaged shortly after lymphocyte activation. This premise is supported by a number of lines of evidence, one of the most compelling being that there is a rapid transient burst in adenylate cyclase activity within minutes after lymphocyte treatment with mitogens or phorbol ester plus calcium ionophore [5, 11–15]. Equally important is the fact that the cAMP-activated kinase, PKA, is critical to the regulation of the cell cycle, a process that is essential for the differentiation of leukocytes into effector cells. This relationship between PKA and cell cycle regulation is based on a number of reports that have shown that inhibition of PKA leads to cell cycle arrest [28–30]. Consistent with a disruption of the cAMP/PKA signaling pathway, cannabinoid compounds have been widely shown to exert anti-proliferative effects in lymphoid cells (reviewed in Refs. 6 and 31). Further evidence implicating a mechanism for immune suppression that involves the inhibition of adenylate cyclase by cannabinoids has been the observation that membrane-permeable cAMP analogs (i.e. dibutyryl cAMP and 8-bromo-cAMP) as well as the pretreatment of leukocytes with pertussis toxin, which blocks the inhibition of adenylate cyclase by cannabinoids, can attenuate many of the immunoinhibitory effects produced by this class of compounds including those produced on humoral immune responses, proliferation, and nitric oxide production by monocytes [5, 32]. Recently, our laboratory has demonstrated in the murine T-cell line EL4.IL-2, that cannabinoids negatively regulate CRE binding proteins [22]. As in the present studies with splenocytes, inhibition of CRE binding by Δ^9-THC in EL4.IL-2 cells was correlated closely with a concomitant inhibition of both adenylate cyclase and PKA activity. This finding indicates that the negative regulation of nuclear protein binding to CRE is not unique to the murine-derived thymoma (i.e. EL4.IL-2) and can be extended to primary leukocytes. Furthermore, as shown in Fig. 2, Δ^9-THC does not directly inhibit PKA activity by interacting with the kinase itself since kinase activity was unaffected by the cannabinoid in the presence of exogenous cAMP. Rather, inhibition of PKA is mediated indirectly through a decrease in cAMP formation resulting from the inhibition of adenylare cyclase following ligand binding to cannabinoid receptors [5, 10].

In the present series of studies, we investigated the modulation of binding activity of nuclear factors specific for CRE in mouse splenocytes following a variety of stimuli in the presence and absence of Δ^9-THC. As is demonstrated by the gel shift studies, forskolin, anti-CD3, or PMA plus

ionomycin all induced a rapid activation of CRE binding. Moreover, consistent with a cAMP/PKA-dependent mechanism for the regulation of the CREB/ATF family of nuclear factors, Δ^9-THC treatment decreased the binding activity to a CRE consensus sequence in a time-related manner in splenocytes activated by each of the three stimuli. Similarly, Δ^9-THC inhibited forskolin-induced CRE binding activity in a concentration-related manner.

Although little is known with respect to the functional significance of altered binding of *trans*-activating factors to CRE sites in the regulation of immunological responses, several recent studies have provided some insight into the role of CRE binding proteins on T-cell regulation. Studies in our laboratory using EL4.IL-2 cells have demonstrated a marked inhibition in PMA plus ionomycin-induced IL-2 expression following cannabinoid treatment. It is notable that this inhibition of IL-2 correlated well with a decrease in CRE-protein binding [22]. In these same experiments, IL-2 expression was found to be similarly inhibited in primary splenocytes by cannabinoid treatment. Although interesting, this adverse regulation of IL-2 through the inhibition of CRE binding was initially puzzling in light of the fact that the regulatory region of the IL-2 gene does not possess any known CRE sites [33]. However, it has been known for some time that the CREB/AFT family of transcription factors, which is best characterized for binding to CRE [34, 35], can form dimers with Fos and Jun [36, 37]. Through the formation of cross-family dimers, nuclear binding proteins expand their repertoire of binding specificity and provide an additional mechanism for cross-talk between signaling pathways. This relationship has been established recently by Chen and Rothenberg [38] where they identified the binding of cross-family dimers, specifically CREB/Fos and CREB/Jun, to the AP-1 proximal (AP-1p) site within the IL-2 promoter. These results are highly consistent with our studies of IL-2 regulation in EL4.IL-2 cells in the presence of cannabinoids. Concordant with a role for cAMP signaling in the regulation of IL-2, we also previously demonstrated by gel shift assays that concomitant stimulation of EL4.IL-2 cells with forskolin and PMA plus ionomycin enhances the magnitude of AP-1p binding as compared with PMA plus ionomycin in the absence of forskolin [22]. Along these same lines, by implicating a positive regulatory role for the cAMP signaling cascade in the regulation of this AP-1p binding in T-cells, it is now understandable why Δ^9-THC was capable of markedly inhibiting PMA/ionomycin-induced AP-1p binding activity. In light of this, we have interpreted our findings in the context of the Chen and Rothenberg results as being indicative that cannabinoid treatment decreases PKA-mediated activation/phosphorylation of CREB proteins, which in turn leads to a decrease in CREB/Fos and CREB/Jun dimers that bind to AP-1p sites in the IL-2 promoter. Equally important with respect to the transcriptional regulation of this cytokine was the finding that even though two AP-1-like sites exist in the minimal essential portion of the IL-2 promoter, that being the AP-1 distal site (AP-1d) and the AP-1p site [33], it is the AP-1p site that exhibits the greatest modulation of binding following phorbol ester plus calcium ionophore in the presence of cAMP activators (i.e. forskolin or IL-1) [39]. In light of the marked inhibition that cannabinoids induced on AP-1p binding activity in EL4.IL-2 cells, it is not surprising that IL-2 transcription is inhibited significantly by Δ^9-THC [22, 40].

Equally provocative have been the recent studies by Barton and coworkers [30] in which they developed a transgenic mouse expressing a dominant negative form of CREB under the specific control of the T-cell specific CD2 promoter/enhancer. CREB in the transgenic mouse T-cells lacked a serine residue which, when phosphorylated, activates this nuclear binding factor. Moreover, this modified form of CREB was incapable of activating CRE-regulated genes. Interestingly, T-cell development appeared to be normal in these transgenic mice. In contrast, thymocytes and T-cells from the CREB transgenic mice exhibited a profound inhibition of proliferative responses to a variety of T-cell stimuli (PMA plus ionomycin, anti-CD3, and concanavalin A), greater than 99% inhibition of IL-2 production, and a G_1 cell-cycle arrest. Furthermore, activated T-cells from these transgenic mice exhibited decreased induction of c-*jun*, c-*fos*, *Fra-2* and *FosB*, which code for AP-1 associated proteins. This decrease is likely due, at least in part, to the fact that some of these genes (i.e. c-*jun*, c-*fos*) are known to possess CRE sites within their own regulatory regions. These findings provide an additional explanation for decreased binding to the AP-1p site in cannabinoid-treated EL4.IL-2 cells. The most striking aspect of the Barton studies, as they relate to our own, pertains to the similarly in functional anomalies between the T-cells possessing aberrant CREB from the transgenic mice and cannabinoid-treated T-cells (i.e. inhibition of IL-2 expression and lymphocyte proliferation to mitogenic stimuli).

One puzzling aspect is that in the Barton studies the authors referred to one set of experiments in which CREB phosphorylation was not blocked in normal T-cells with the PKA inhibitor H-8. Based on this finding, the authors questioned whether CREB activation is mediated by PKA. Unfortunately, because the data were not shown for this experiment, it is unclear how the studies were, in fact, performed. Nonetheless this finding is intriguing in light of a number of well-defined studies which have established that although not solely regulated by PKA, the primary activator of the CREB/ATF family of transcription factors is this cAMP-activated kinase [21, 34, 35]. This has been most extensively established for CREB, which is phosphorylated by PKA on serine 133 to confer DNA binding activity on this protein [21].

In summary, our results show that inhibition of the cAMP signaling cascade by Δ^9-THC treatment of splenocytes led to a significant decrease in the binding of *trans*-activating factors to CRE sites. We believe that this inhibition of CRE binding is a critical step in the mechanism responsible for the immunoinhibitory effects induced

by cannabinoid compounds. Not only does this decrease in the activation of CREB/ATF family proteins adversely affect the expression of IL-2, but likely also decreases the expression of the immediate early genes, specifically those belonging to the Fos and Jun family as shown by Barton and coworkers with T-cells possessing a mutant CREB. The importance of CRE binding is further supported by the fact that we and others have shown its rapid induction in primary leukocytes in response to activational stimuli such as anti-CD3 or PMA plus ionomycin [22, 30, 38].

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