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Regulation of the cAMP cascade, gene expression and immune function by cannabinoid receptors

Norbert E. Kaminski *

Department of Pharmacology and Toxicology and Department of Pathology, B-330 Life Sciences Building Michigan State University, East Lansing, MI 48824, USA

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Abstract

The objective of this article is to discuss the putative role of cannabinoid receptors in immune modulation by cannabinoid compounds. The primary focus is on the signal transduction events that are initiated following ligand binding to cannabinoid receptors and how these events lead to detrimental effects on the normal responsiveness of immunocompetent cells. Toward this end, signalling events are traced from the cannabinoid receptor to the transcription factors which are adversely regulated in the presence of cannabinoid compounds during leukocyte activation. Moreover, this aberrant regulation of transcription factors is discussed in the context of altered gene expression and the impact this has on leukocyte function. Lastly, an important goal of this article is to dispel a long standing myth that the cyclic adenosine 3′:5′-monophosphate (cAMP) cascade is a negative regulatory pathway for immunocompetent cells. This chapter examines two major immunologic cell-types which are well established as exhibiting altered function following cannabinoid treatment, helper T-cells and the macrophage. Not discussed are the effects of cannabinoids on B-cell function. This is primarily due to the rather refractory nature of B-cells to inhibition by cannabinoids in spite of the fact that this cell-type expresses functional cannabinoid receptors [Schatz, A.R., Koh, W.S., Kaminski, N.E., 1993. Δ^9-tetrahydrocannabinol selectively inhibits T-cell dependent humoral immune responses through direct inhibition of accessory T-cell function. Immunopharmacol., 26, pp. 129–137.]. One cautionary note, although the focus of this article is on cannabinoid receptor mediated signalling events, immune modulation by cannabinoid compounds is likely multi-factorial presumably involving receptor as well as receptor-nonrelated events. Effects on leukocytes by cannabinoids which are believed to be mediated by receptor-nonrelated events are outside the scope of this paper and will not be discussed. One last introductory point is that even though their is presumably little overlap in the genes which are regulated by cannabinoids in leukocytes as compared to other cell-types (e.g., neural cells), the major signalling pathways involved in cellular regulation are ubiquitous. With that in mind, it is likely that their is a considerable amount of similarity in the signalling pathways regulated by cannabinoids in cell-types of different lineage, given that they express cannabinoid receptors. In this context, signalling events observed in leukocytes can provide important insight into which genes may be modulated by cannabinoid in other cell types. © 1998 Elsevier Science B.V.

Keywords: Immune modulation; Gene expression; Cannabinoid receptors

1. Introduction

Historically, the mechanism by which cannabinoids produce their broad array of physiological effects has been attributed to nonspecific intercalation of these lipophilic compounds into the lipid bilayer of the cell membrane resulting in the disruption of membrane processes. Although the specific mechanism(s) of action for cannabinoids still remains to be fully elucidated, a number of distinct lines of evidence have supported the involvement of receptors. Many of the early observations were made by using the central nervous system (CNS) and CNS-derived cell-lines as models. One of the most compelling is the

Abbreviations: Δ^9-THC, delta-9-tetrahydrocannabinol; G-protein, guanine-nucleotide-binding protein; PMA, phorbol-12-myristate-13-acetate; Io, ionomycin; AFC, antibody forming cell; bp, base pair(s); RT-PCR, reverse transcription-polymerase chain reaction; CB1, cannabinoid receptor type 1; CB2, cannabinoid receptor type 2; iNOS, inducible nitric oxide synthase; cAMP, cyclic adenosine 3′:5′-monophosphate; CRE, cAMP response element; NF-κB, nuclear factor for immunoglobulin κ chain in B-cells; LPS, lipopolysaccharide; sRBC, sheep erythrocytes; IgM, immunoglobulin M; NO, nitric oxide; PKA, protein kinase A; PKC, protein kinase C; IL-1, interleukin-1; IL-5, interleukin-5; CREB, cAMP response element binding protein; CREM, cAMP response element modulator; ATF, activating transcription factor; NF-AT, nuclear factor of activated T-cells; CHO, chinese hamster ovary cells; CNS, central nervous system

* Tel.: +1 517 3533786; fax: +1 517 3538915.

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general lack of correlation between the degree of lipophilicity of specific cannabinoid congeners and their biologic activity (Thomas et al., 1990). Moreover, early studies demonstrated negative regulation of adenylate cyclase following exposure to cannabinoids, an enzyme almost exclusively associated in mammalian systems with membrane bound receptors (Howlett, 1985; Howlett and Fleming, 1984). The above also correlates well with binding studies which demonstrated specific and saturable binding by cannabinoids in brain synaptosome preparations (Harris et al., 1978). Lastly, a novel guanine-nucleotide-binding protein (G-protein) coupled receptor was cloned from a rat brain cDNA library which exhibited stereospecific cannabinoid binding and negative regulation of adenylate cyclase when transfected into chinese hamster ovary cells (CHO) cells in the presence of cannabinoid compounds (Matsuda et al., 1990). Since the identification of a cannabinoid receptor in neuronal tissues (cannabinoid receptor type 1 (CB1)), a second major form of the receptor (cannabinoid receptor type 2 (CB2)) has been isolated and cloned from the promyelocytic line HL60 (Munro et al., 1993). The two receptors share approximately 68% identity within their transmembrane regions, that portion of the receptor believed to possess the ligand binding domain (Munro et al., 1993). In spite of the marked differences in identity, most cannabinoid compounds bind with similar affinity to both CB1 and CB2. One exception to this rule is the plant derived cannabinoid, cannabinol, which possesses significantly greater binding affinity for CB2 than for CB1 (Munro et al., 1993); exhibits good binding affinity to mouse spleen cells (Schatz et al., 1997); and has immunomodulatory activity in a number of leukocyte preparations (Condie et al., 1996).

2. CB1 and CB2 receptor distribution

Shortly following the identification of CB1 in rat brain, CB1 was also identified in human brain (Gerard et al., 1990) and human testis (Gerard et al., 1991) and found to be highly conserved between these two as well as other species. The first identification of cannabinoid receptors within the immune system was in mouse spleen cells (Kaminski et al., 1992). This cellular preparation exhibited: (a) saturable specific binding of the high affinity cannabinoid receptor radioligand, [^{3}H]-CP-55940 with K_d values in the high picomolar range; (b) presence of RNA transcripts for CB1, as demonstrated by reverse transcription-polymerase chain reaction (RT-PCR); (c) stereoselective inhibition of the antibody forming cell (AFC) responses; and (d) a marked inhibition of adenylate cyclase following cannabinoid treatment (Schatz et al., 1992; Kaminski et al., 1992). Interestingly, CB1 is the primary form of the cannabinoid receptor within the CNS but is modestly expressed within the immune system (Bouaboula et al., 1993; Kaminski et al., 1992; Schatz et al., 1997). CB1 RNA has been detected in human B-cells, T-cells and monocytes (Bouaboula et al., 1993), and in mouse spleen (Kaminski et al., 1992) but is below the level of quantitation in mouse thymus even as assayed by RT-PCR (Schatz et al., 1997). Conversely, CB2 appears to be the predominant form of cannabinoid receptors within the immune system with no quantifiable expression within the brain as determined by Northern analysis (Munro et al., 1993) and quantitative RT-PCR (Schatz et al., 1997). CB2 expression has been demonstrated in rat spleen (Munro et al., 1993), mouse spleen and thymus (Schatz et al., 1997), and a number of immune system-derived cell lines including the T-cell lines, EL4.IL-2 (Condie et al., 1996), HPB-ALL, Jurkat E6-1 (Schatz et al., 1997), the monocytic lines, HL60 (Munro et al., 1993) and RAW264.7 (Jeon et al., 1996), and the mast cell line, RBL-2H3 (Facci et al., 1995). Extensive mapping of CB1 and CB2 receptor protein expression in primary tissues is awaiting the development of receptor specific antibodies.

One interesting aspect about the CB2 receptor is that CB2 mRNA transcripts in mouse lymphoid tissues ($\sim$ 4 kb) (Condie et al., 1996; Schatz et al., 1997) are significantly larger than that observed in rat spleen and man (2.5 kb) (Munro et al., 1993). It is likely that the different-sized RNA transcripts in the mouse reflects a possible mutation at the polyadenylation site or perhaps even alternative splicing in the mouse CB2 gene. Recently, Shire et al. (1995) demonstrated alternative splicing in the CB1 gene. A shorter CB1 sequence, CB1A, was cloned from IM-9 cells that lacks a 167-base pair intron within the sequence encoding the amino-terminal tail of the receptor which is expressed, along with CB1, in a wide range of tissues and cell lines. The functional significance of this alternative form of the CB1 receptor is presently unclear.

3. Regulation of adenylate cyclase by cannabinoid compounds

As initially suggested by Howlett et al. (1986) from studies in neuronal cell lines prior to the identification of cannabinoid receptors, both CB1 and CB2 negatively regulate adenylate cyclase activity through a pertussis toxin sensitive GTP-binding protein (Kaminski et al., 1992). Modulation of adenylate cyclase by cannabinoid compounds has been compellingly demonstrated in virtually every tissue and cell-line shown to express functional cannabinoid receptors as well as in cell-lines devoid of either CB1 and CB2 but successfully transfected with one of the two receptor genes (i.e., CHO cells) (Bayewitch et al., 1995; Matsuda et al., 1990; Sliptz et al., 1995). With respect to the immune system, inhibition of adenylate cyclase activity by cannabinoid compounds has been demonstrated in mouse splenocytes (Schatz et al., 1992), purified mouse splenic T- and B-cells (Schatz et al., 1997), and in a number of immune system derived cell lines

including the T-cell lines, EL4.IL-2 (mouse) (Condie et al., 1996) and HPB-ALL (human) (Schatz et al., 1997) and the monocytic lines, RAW264.7 (Jeon et al., 1996). It is notable that the human T-cell line, Jurkat E6-1, is refractory to modulation of adenylate cyclase by cannabinoid compounds. Interestingly, this specific clone of the Jurkat line also expresses aberrant sized CB2 mRNA transcripts (approximately 2.3, 4.5 and 5.7 kb) and possess no detectable CB1 as assayed by Northern analysis of poly(A) RNA (Schatz et al., 1997). Southern analysis of genomic DNA following digestion with a variety of restriction enzymes known to cut within the CB2 gene revealed no significant difference from human genomic DNA (Schatz et al., 1997). This suggests that in Jurkat E6.1 cells, CB2 mRNA is likely processed in an aberrant fashion resulting in the expression of nonfunctional CB2 receptors. More importantly, this series of studies with Jurkat E6.1 cells helped to demonstrate that modulation of adenylate cyclase activity by cannabinoids is not mediated through nonspecific actions and is dependent on functional cannabinoid receptor expression.

4. Does the inhibition of adenylate cyclase by cannabinoids have any relevance to immune function?

As discussed above, one of the earliest signalling events initiated by ligand binding to cannabinoid receptors is the inhibition of adenylate cyclase which leads to a decrease in the production and accumulation of intracellular cyclic adenosine 3′:5′-monophosphate (cAMP). That this, in turn, leads to an inhibition of immune function is contrary to a long held immunologic axiom, that the primary role of the cAMP signalling cascade in immune function is to serve as a negative regulatory pathway. This premise is based on many past, as well as recent, studies in which immunocompetent cells have been shown to exhibit decreased function when treated with high concentration (>100 μM) of membrane permeable cAMP analogs (e.g., dibutyryl-, 8-bromo–cAMP). This immune inhibition has been demonstrated in B- and T-cells (Hsueh and Lai, 1995; Johnson et al., 1988; Tsuruta et al., 1995) and most often is also associated with an arrested in cell cycle progression (Maraguchi et al., 1984). The latter observation is especially intriguing in light of the recent finding that cell cycle progression appears to be dependent on protein kinase A (PKA) as demonstrated in a Xenopus model system. The aforementioned studies show that: (1) inhibition of PKA induces cell cycle arrest while simultaneously blocking the degradation of the M phase promoting factor, cyclin B-p34^{cdc2} complex; (2) intracellular cAMP concentrations and PKA activity coordinately increases and decreases at specific phases of the cell cycle; (3) PKA activators including low cAMP concentrations (<50 μM) of 8-bromo–cAMP, reverse cell cycle arrest induced by a recombinant PKA regulatory subunit which blocks endogenous PKA activation; and most relevant to this discussion, (4) cAMP at concentrations >50 μM markedly inhibits cell cycle progression (Grieco et al., 1996). The authors speculated that high cAMP concentrations are inhibitory to cell cycle most likely due to nonspecific phosphorylation that occurs in the presence of nonphysiological amounts of PKA activity. Not surprisingly and without exception, all of the studies cited above demonstrating an inhibition of immune function by cAMP were performed either using high concentrations (>250 μM) of membrane permeable cAMP analogs or agents that induced a high level of intracellular cAMP.

In contrast to the aforementioned reports, an increasing number of studies have shown convincingly not only a positive but in many cases an obligatory role for cAMP as a mediator of cellular responses in immunocompetent cells. From these findings it is now clear that to generalize that the cAMP signalling pathway is strictly involved in negative regulation is an oversimplification. It is notable that the same cAMP analogs which have been shown to be inhibitory at high concentrations, are immunostimulatory at lower, more physiologically relevant, concentrations (<100 μM) in a variety of assay systems (Kaminski et al., 1994; Kammer, 1988; Koh et al., 1995). The critical role of cAMP in lymphoid cell function is further supported by the fact that there is a rapid transient burst in adenylate cyclase activity within the first 5 min following lymphocyte activation by mitogens or phorbol ester plus calcium ionophore (Hadden et al., 1972; Kaminski et al., 1994; Pepe et al., 1994; Russell, 1978; Smith et al., 1971; Watson et al., 1994) implicating positive lymphocyte regulation through this mechanism. The relevance of this rise in cAMP as it applies to specific helper T-cell and macrophage functions will be discussed in more detail below. Moreover, it is clear that the inhibitory effects produced by cannabinoids on immunocompetent cells can be abrogated by blocking or reversing the cannabinoid-induced decrease in intracellular cAMP. This has been demonstrated in several ways. Antibody responses requiring helper T-cells and macrophages as accessory cells, as in the case with the T-cell dependent antigen, sheep erythrocytes (sRBC), are markedly sensitive to inhibition by cannabinoid compounds (Schatz et al., 1993). The inhibition of this response can be abrogated in vitro by direct addition of low concentrations (50–100 μM) of membrane permeable cAMP analogs (i.e., dibutyryl- or 8-bromo–cAMP) to the cell cultures. However, this reversal of the AFC responses can only be achieved if the cAMP analogs are added to culture within the first 30–60 min following antigen stimulation (Kaminski et al., 1994). This is consistent with the hypothesis that cannabinoids inhibit an early T-cell mediated activation event and/or interfere with macrophage–T-cell interactions. It is important to emphasize that as described by many laboratories, these studies also demonstrated that high concentration (>100 μM) of either dibutyryl–cAMP or 8-bromo–cAMP, alone, are

markedly inhibitory on spleen cells with concomitant changes in cell viability occurring at or above 250 μM concentrations (Kaminski et al., 1994). Similarly, cannabinoid-mediated inhibition of the anti-sRBC antibody response as well as inhibition of lymphocyte proliferation induced by phorbol ester and calcium ionophore can also be blocked by pretreating immunocompetent cells overnight with pertussis toxin, an agent that ADP-ribosylates GTP-inhibitory proteins. By doing so, the transduction of signalling from the receptor to adenylate cyclase via GTP-binding proteins is blocked. Likewise, cannabinoid inhibition of the anti-sRBC antibody response can also be attenuated by concomitant treatment of immunocompetent cells with the hormone, glucagon (Koh et al., 1996). Glucagon functions by binding to G-protein coupled receptors which positively regulate adenylate cyclase thus increasing intracellular cAMP. In this case the inhibition of cAMP formation by delta-9-tetrahydrocannabinol (Δ^9-THC) is balanced by an increase in intracellular cAMP formation initiated through glucagon receptors. Similarly treatment of spleen cells with the adenylate cyclase inhibitor, dideoxyadenosine, produces a marked inhibition of the anti-sRBC response as previously demonstrated with cannabinoids (Koh et al., 1996). And as demonstrated with cannabinoid treatment, dideoxyadenosine-mediated inhibition is also partially reversed by glucagon. Cannabinoids also inhibit lipopolysaccacharide (LPS)-induced nitric oxide (NO) formation by mouse-derived macrophages and RAW 264.7 cells, a macrophage-derived line (Jeon et al., 1996). The inhibition of this macrophage-mediated response can also be abrogated by concomitantly treatment of the cells with 8-bromo–cAMP (50–100 μM) as will be discussed later on in this article. All of the examples described above are consistent with a positive regulatory role for the cAMP signalling cascade in immune cell function and with this signalling pathway being the primary intracellular target responsible for immune disregulation by cannabinoids.

5. The role of the cAMP signalling cascade in T-helper cell function

In the remainder of this chapter, the discussion will primarily focus on the role of the cAMP signalling cascade in cannabinoid-mediated disruption of two immunological responses: (a) interleukin-2 (IL-2) expression by T-cells; and (b) inducible nitric oxide synthase (iNOS) expression by macrophages. We will first focus on the disregulation of interleukin-2 (IL-2) by cannabinoids in helper T-cells.

Recently, studies have focused on the effects exerted by cannabinoids, through an inhibition of the cAMP cascade, on lymphokine production by T-cells (Nakano et al., 1993; Condie et al., 1996). Specifically, the regulation of IL-2, which is a critical mediator of T-cell clonal expansion, was investigated. In these studies the extensively characterized IL-2 secreting murine-derived thymoma, EL4.IL-2, was used as a model system. This cell-line expresses CB2 receptors but not CB1 and exhibits inhibition of forskolin-stimulated cAMP accumulation following cannabinoid treatment which is indicative that CB2 is in fact functionally expressed within this cell-line (Condie et al., 1996). As illustrated in Fig. 1, the cAMP cascade is regulated by the formation of cAMP which binds to the regulatory subunits of PKA resulting in the release and activation of PKA-catalytic subunits. These catalytic subunits go on to phosphorylate a variety of intracellular proteins including the cAMP response element binding protein/activating transcription factor (CREB/ATF) family of transcription regulators which consist of CREB, ATF and cAMP response element modulator (CREM). CREB, which is the best characterized member of this family (and used for illustrative purposes in Fig. 1), is activated by PKA-mediated phosphorylation at Ser residue 133 (Gonzalez and Montminy, 1989) and forms either homo- or heterodimers with a variety of other transcription factors capable of binding cAMP regulatory element (CRE) DNA sequences present in the promoter region of a variety of genes. Upon stimulation of EL4.IL-2 cells with forskolin in the presence of Δ^9-THC, PKA is markedly inhibited. Moreover, examination of CREB/ATF family member activation by gel shift assays, using a cAMP response element (CRE) consensus motif, demonstrated that forskolin treatment significantly upregulates DNA binding by 30 min which exhibits peak binding activity at 60 min followed by a rapid decrease at 90 min. This finding is consistent with a rapid activation of the cAMP cascade following forskolin-treatment. In the presence of cannabinol, CREB/ATF binding was found to be inhibited at all of the time points described above (Condie et al., 1996). It is notable that although the mechanism for the transient activation of CRE DNA binding, even in the presence of forskolin which provides sustained activation of cAMP, has not been fully characterized, recently the Ser/Thr protein phosphatase, PP-1, which is activated by PKA, has been reported to be a major down-regulator of CREB activity following cAMP stimulation (Hagiwara et al., 1992). Other authors and us (Hagiwara et al., 1992; Koh et al., 1997) have observed that this decrease in CRE binding at later time points ($\geq$ 90 min) following forskolin stimulation in the absence of cannabinoid treatment is blocked by okadiac acid, a protein phosphatase inhibitor.

Measurements of IL-2 expression following phorbol-12-myristate-13-acetate/ionomycin (PMA/Io) stimulation in EL4.IL-2 cells as well as primary mouse splenocytes, in the presence of either Δ^9-THC or cannabinol, showed a marked inhibition of IL-2 secretion which was closely correlated with a significant decrease in IL-2 gene transcription (Condie et al., 1996). This finding was initially intriguing in light of the fact that there are no CRE regulatory motifs in the promoter region of the IL-2 gene. It is known that IL-2 gene transcription is highly regulated through a number of well characterized recognition sites in

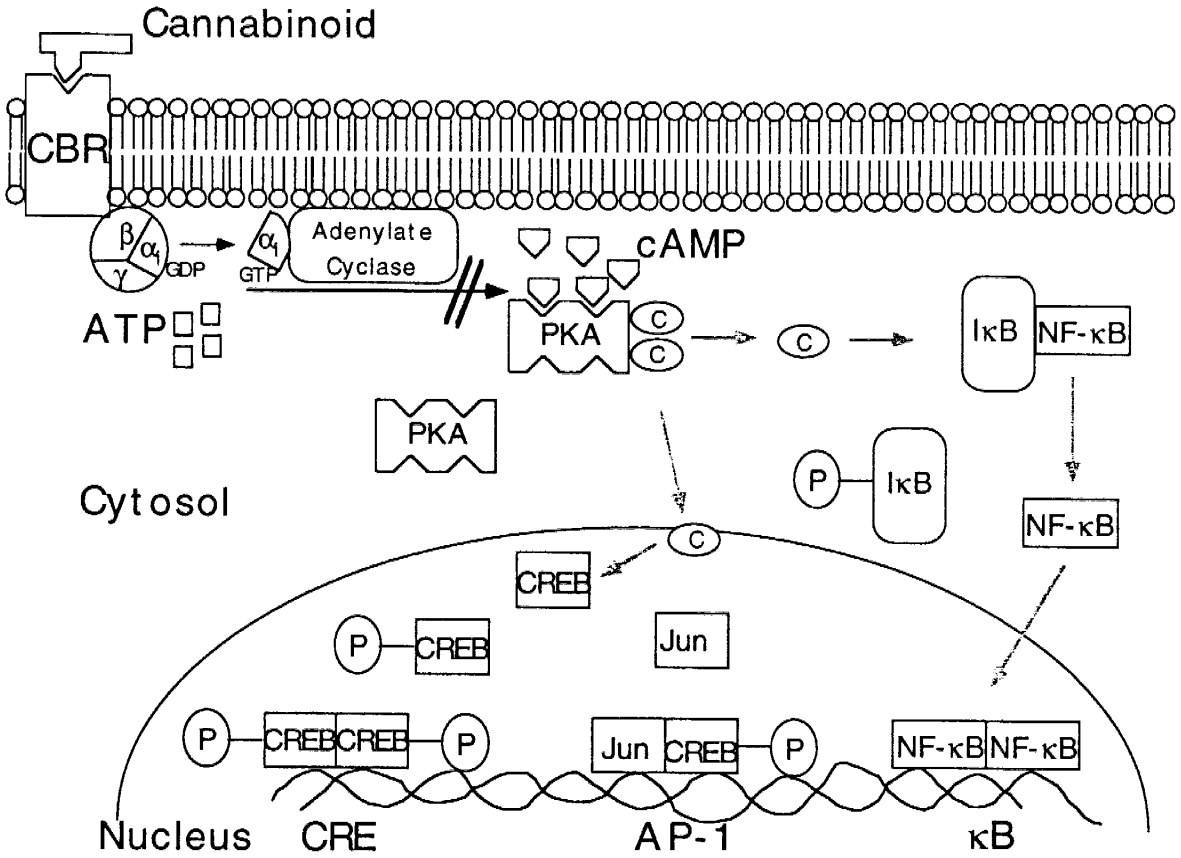

Fig. 1. The putative signaling pathway associated with cannabinoid receptors (CBR). Ligand binding to CBR induces the interaction of $G\alpha i$ with adenylate cyclase leading to a decrease in the conversion of ATP to cAMP. Decreased cAMP formation inhibits the activation of protein kinase A (PKA) and the release of PKA catalytic (C) subunits. This inhibition of PKA activation in turn inhibits the phosphorylation of IκB, the cognate inhibitor of NK-κB/Rel, blocking NK-κB/Rel activation, translocation and DNA binding. Inhibition of PKA also blocks the nuclear translocation of PKA catalytic subunits which phosphorylate/activate CREB/ATF transcription factors that bind to CRE motifs. Lastly, CREB/ATF family members also form dimers with Fos and Jun and are capable of binding to AP-1 motifs (e.g., AP-1 proximal site in the IL-2 promoter). Therefore inhibition of adenylate cyclase can result in decreased binding by *trans*-acting factors to CRE, AP-1 and κB motifs.

the promoter region for inducible and noninducible regulatory factors. The inducible factors include AP-1, nuclear factor of activated T-cells (NF-AT), nuclear factor for immunoglobulin κ chain in B-cells (NF-κB), and CD28RE. Interestingly, forskolin has been demonstrated by gel shift assays, in EL4 cells, to enhance phorbol ester/calcium ionophore-induced AP-1 binding in the IL-2 promoter (Novak et al., 1990). Moreover, several laboratories have also shown that both Fos and Jun family members can dimerize with CREB and that these 'chimeric' heterodimers in turn are capable of binding to AP-1 sites (Hai and Curran, 1991; Ivashkiv et al., 1990). Recently, this phenomenon has been demonstrated employing anti-CREB and anti-Fos/Jun gel shifts which identified all three protein types bound to an AP-1-like site (AP-1 proximal site: AP-1p) in the IL-2 promoter, suggesting that CREB family proteins help to regulate IL-2 transcription through the formation of heterodimers with Fos and Jun (Chen and Rothenberg, 1993). Concordant with a role for CREB protein binding at the AP-1p site in the IL-2 promoter, cannabinol markedly inhibited PMA/Io-induced AP-1p binding in EL4.IL-2 cells (Condie et al., 1996). Interestingly, this convergence of the PKA and protein kinase C (PKC) regulatory pathways is further supported by the fact that forskolin augmented PMA/Io-induced AP-1p binding in this same cell line (Condie et al., 1996). In addition to the disregulation of IL-2 expression by cannabinoids through an inhibition of nuclear regulatory factor binding at AP-1 sites, we have also observed that cannabinoids markedly inhibit the activation of NF-κB in a variety of T-cell models as well as in the macrophage line, RAW264.7 (Jeon et al., 1996). This is likely an additional contributing factor to the inhibition of IL-2 gene expression.

A positive role by modest transient increases in cAMP during T-cell activation is consistent with the above discussion as well as the recent finding that unlike for a variety of cell types including fibroblasts, adipocytes, and muscle cells in which cAMP antagonizes the Raf–MAP kinase pathway (Burgering et al., 1993; Cook and McCormick, 1993; Graves et al., 1993; Hafner et al., 1994; Hordijk et al., 1994; Sevetson et al., 1993; Wu et al., 1993)

in T-cells, this pathway is resistant to negative influences normally associated with short-term increases in cAMP (Hsueh and Lai, 1995). This is evidenced by the fact that cAMP does not inhibit ERK2, the dominant form of MAP kinase in T-cells (Hsueh and Lai, 1995). Similarly, the newly identified c-Jun N-terminal kinase which defines a T-cell antigen receptor independent activation pathway is also resistant to short-term increases (< 30 min) in cAMP (Hsueh and Lai, 1995). Conversely, sustained (2 h) treatment of T-cells with 0.5 mM dibutyryl–cAMP induces an antagonism of c-Jun N-terminal kinase which is not due to a decrease in the synthesis of the kinase but is dependent on protein synthesis (Hsueh and Lai, 1995).

As already mentioned, the accessory function played by helper T-cells in antibody responses is especially sensitive to inhibition by cannabinoid compounds. This observation is very consistent with recent findings which suggest that the cAMP signaling cascade may serve as a switching mechanism between the helper T-cell subpopulations, Th1 which facilitate cell-mediated immune responses, and Th2, that subtype which regulates humoral immune responses. Several recent studies indicate that high intracellular concentrations favor Th2 responses. For example, high concentrations of cAMP (1 mM) activate the binding of nuclear factors to GATA-3 and the conserved lymphokine element (CLE0) to enhance the expression of the B-cell differentiation factor, interleukin-5 (IL-5) (Lee et al., 1995). Moreover, high sustained concentrations of cAMP inhibit IL-2 expression by Th1 cells (Siegel et al., 1995). This coupled with the fact that T-cells show a marked inhibition in a number of responses following cannabinoid treatment, we have proposed that cAMP is essential for both Th1 and Th2 lymphokine gene expression; however, low and perhaps transient intracellular cAMP concentrations favor the activation of Th1 lymphokines, whereas, high and sustained cAMP concentrations appear to favor the induction of Th2 associated cytokines. Further, the apparent requirement for high cAMP concentrations for IL-5 expression provides an explanation for the marked sensitivity of T-cell dependent humoral responses to inhibition by cannabinoids since these compounds are potent inhibitors of adenylate cyclase.

6. The role of the cAMP signaling cascade in the regulation of iNOS in macrophages

Recently, it has been demonstrated that cannabinoids inhibit the induction of iNOS gene expression in LPS-stimulated macrophages (Jeon et al., 1996). The iNOS catalyzes the production of large amounts of NO from L-arginine and molecular oxygen (Palmer et al., 1988). Expression of iNOS is rapidly induced in macrophages by LPS which is a major constituent of gram negative bacterial cell membranes. The production of NO in turn contributes to the cytolytic function of macrophages and is believed to be an important component of the innate immune response (Hibbs et al., 1987). In spite of much current interest in the role of iNOS in host resistance to pathogens and its involvement in the very closely related process, inflammation, its regulation is only partially understood. Much insight pertaining to iNOS regulation as it relates to NO production has recently emerged from the sequencing of the regulatory region of this gene. Interestingly, the promoter region for iNOS contains two κB-binding sites, one at position -79 and a second at position -962 (Lowenstein et al., 1993). Protein binding at the κB-site is necessary to confer iNOS inducibility by LPS (Xie et al., 1994).

The NF-κB/Rel family of transcription factor are pleiotropic regulators of many genes involved in immune and inflammatory responses, including iNOS (Grilli et al., 1993; Xie et al., 1994). In unstimulated cells, NF-κB/Rel proteins remain quiescent in the cytoplasm and bound to their cognate inhibitor, IκB. Activation of macrophages by certain external stimuli induces phosphorylation of IκB by PKA to release the active DNA-binding form of NF-κB/Rel family members to translocate to the nucleus where they bind κB motifs in the regulatory region of a variety of genes (Shirakawa and Mizel, 1989). LPS-treatment of macrophages activates both PKC and PKA, the latter being induced by an elevation in intracellular cAMP (Muroi and Suzuki, 1993), (Novotney et al., 1991). Additionally, interleukin-1 (IL-1), which is induced by LPS, also contributes to the elevation of cAMP. The coordinate activation mediated through LPS and IL-1 is followed by a rapid increase in iNOS expression and nitrite formation (Koide et al., 1993; Alonso et al., 1995). Based on the role played by cAMP signaling in the regulation of NF-κB/Rel proteins and the ability of cannabinoids to inhibit NF-κB DNA binding in lymphoid cell preparations (unpublished observation), we investigated whether cannabinoids would likewise inhibit LPS-induced iNOS expression by macrophages. The results summarized below come from studies using primary mouse peritoneal macrophages and the macrophage-derived line, RAW264.7 (Jeon et al., 1996).

RAW264.7 cells express CB2 but not CB1 RNA transcripts (Jeon et al., 1996). The magnitude of basal CB2 RNA expression, as determined by quantitative RT-PCR is significantly greater in RAW264.7 cells than observed in the previously discussed T-cell line, EL4.IL-2 (Condie et al., 1996; Jeon et al., 1996). Based on a number of studies which are beyond the scope of this article and as suggested by quantitation of CB2 RNA transcripts, it is likely that macrophages express a markedly greater number of CB2 receptors than present on T-cells. However this conclusion will need to be confirmed in isolated and purified primary leukocytes either by Western blotting with CB2 specific antibodies or by radioligand binding analysis with high affinity, CB2 selective, agonists. The treatment of RAW264.7 with Δ^9-THC was found to produce a dose-re-

lated inhibition in forskolin-stimulated cAMP accumulation confirming the functional expression of cannabinoid receptors by the RAW264.7 cell-line. Cannabinoid treatment of RAW264.7 cells also produced a dose-dependent inhibition of LPS-induced NO production which was closely correlated with a decrease in iNOS RNA. A similar inhibition in LPS-induced NO production was also observed in resident (unelicited) mouse peritoneal macrophages. Interestingly, cannabinoid-mediated inhibition of LPS-induced NO production was reversed by concomitant treatment of RAW264.7 cells with 8-bromo–cAMP. Gel shift assays demonstrated that both LPS and forskolin treatment of RAW264.7 cells alone, significantly increased protein binding to κB and CRE DNA motifs. However, nuclear protein binding activity to either CRE or κB was significantly decreased in the presence of Δ^9-THC following either stimulus (i.e., forskolin or LPS). These results further support previous reports that: (a) the cAMP-signaling cascade is a major regulator of NF-κB/Rel family of DNA binding proteins; and (b) NF-κB/Rel activation is required for iNOS gene expression in response to LPS in macrophages.

7. Conclusions

Cannabinoids are well established as being immune modulators. Elucidation of the specific mechanisms responsible from this biologic effect has a number of significant implications. First, from a basic science standpoint, cannabinoids are a very useful class of pharmacologic probes that can be utilized to characterize the signaling events within the cAMP cascade and its role in a variety of biological processes. As discussed above, much has already been gleaned from the use of cannabinoids as experimental probes in determining the role of cAMP signaling in the regulation of two immunological responses, IL-2 secretion by T-cells and NO production by macrophages. Also relevant to basic research but beyond the scope of this discussion is the physiologic role of cannabinoid receptors and their endogenous ligands. Second and perhaps most importantly, cannabinoid compounds have a strong potential of becoming a clinically useful class of immunomodulators. From the studies discussed above, cannabinoids have the ability to inhibit DNA binding of three major families of nuclear transcription factors, CREB/ATF, AP-1 (Fos/Jun) and NF-κB/Rel. All three of these families of *trans*-acting factors are well known to be involved in the regulation of many cytokine and inflammatory mediator genes, with AP-1 (Fos/Jun) and NF-κB/Rel likely being most critical in this respect. Since relatively high concentrations of plant-derived cannabinoids are in fact required to produce many of the immunological effects discussed, one of the primary pharmacologic challenges in developing cannabinoid-based therapeutic agents will be to design high affinity CB2 selective ligands. By doing so, CNS-inactive and immune system specific cannabinoids may possess a significant advantage as immune modulators over presently utilized therapeutic agents such as the systemically acting glucocorticoids.

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