Signal Transduction Activated by Cannabinoid Receptors

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Abstract: Since the discovery that cannabinoids exert biological actions through binding to specific receptors, signal mechanisms triggered by these receptors have been focus of extensive study. This review summarizes the current knowledge of the signalling events produced by cannabinoids from membrane receptors to downstream regulators. Two types of cannabinoid receptors have been identified to date: CB_1 and CB_2 both belonging to the heptahelichoidal receptor family but with different tissue distribution and signalling mechanisms. Coupling to inhibitory guanine nucleotide-binding protein and thus inhibition of adenylyl cyclase has been observed in both receptors but other signal transduction pathways that are regulated or not by these G proteins are differently activated upon ligand-receptor binding including ion channels, sphingomyelin hydrolysis, ceramide generation, phospholipases activation and downstream targets as MAP kinase cascade, PI3K, FAK or NOS regulation.

Cannabinoids may also act independently of CB_1or CB_2 receptors. The existence of new unidentified putative cannabinoid receptors has been claimed by many investigators. Endocannabinoids activate vanilloid TRPV1 receptors that may mediate some of the cannabinoid effects. Other actions of cannabinoids can occur through non-receptor-mediated mechanisms.

1. INTRODUCTION

The term cannabinoid comprises a series of C*annabis sativa*-derived compounds as well as endogenous and synthetic analogs that exert a wide spectrum of biological effects. The major psychotropic component of marijuana is Δ^9-Tetrahydrocannabinol (Δ^9-THC) but more than 60 cannabinoid substances have been isolated from the plant. The three major endogenous cannabinoids identified to date, i. e. anandamide, 2-arachidonyl-glycerol (2-AG) and 2-arachidonyl-glyceryl ether, are arachidonic acid-derived compounds and they represent a novel neuroimmunomodulatory system. These compounds bind to specific receptors that have been named cannabinoid receptors (CB). Two types of cannabinoid receptors, CB_1 and CB_2, have been identified and emerging evidences suggest the existence of additional receptors. The CB_1 cannabinoid receptor has been cloned from mammals including rat [1], human [2], mouse [3, 4] and cat [5], and from other vertebrates as birds [6], amphibians [7], and fish [8] exhibiting a high degree of identity between species, ranking from 99% to 72%. CB_1 is highly expressed in central nervous system preferentially in hippocampus, striatum, substantia nigra, and cerebellar cortex [9-11], and it has been detected not only in neurones but also in astrocytes [12] and microglial cells [13,14]. There are increasing evidences that CB_1 is expressed in peripheral tissues and it has been found in gastrointestinal tract [15-18], urinary bladder [19], lung [20], some immune tissues as spleen [21] and thymus [22], endocrine organs as thyroid [23, 24], adrenal gland [22, 24], testis [2, 22, 25], ovary [22, 24], in other reproductive tissues as uterus [22, 26] and prostate [27] and in rat adipose tissue [28]. The CB_2 cannabinoid receptor has been cloned from human [29], mouse [30] and rat [31, 32] the latter two sharing 82% and 81% of their nucleic acid identity respectively with the human CB_2. Tissue distribution of CB_2 is different from that of CB_1. CB_2 is located mainly in immune tissues and cells [22, 33], although it has been also found in retina [34], skin [35] and some malignant cells [36]. Human CB_1 and CB_2 receptors share 44% overall aminoacid identity. A new putative cannabinoid receptor gene has been recently identified in the invertebrate *Ciona intestinalis* that share 28% sequence identity with the human CB_1 and 24% sequence identity with the human CB_2 [37]. The structure of CB_1 and CB_2 is consistent with heptahelical receptor family which exert most of their signal activation by coupling to G-proteins [38].

The endo-cannabinoid system has been implicated in two major biological roles i.e. modulation of neurotransmitter release and modulation of immune functions. Endogenous cannabinoids mediate retrograde signalling in neuronal tissues that may be involved in the inhibition of the release of different excitatory or inhibitory neurotransmitters [39, 40 and reviewed in 42-44]. Cannabinoids has been demonstrated to inhibit the evoked release of Ach, GABA, NA, DA, 5-HT, Glu, Gly, d-Asp, and CCK [reviewed in 45], which may explain the complex neurobehavioral effects produced by this drugs. The effect of cannabimimetic agents on the function of immune cell function is still unclear and requires further elucidation but they produce a deleterious effect on the immune response causing the impairment of macrophage functions, perturbation of immunoglobulin production and down-regulation of immune cells activity [46]. The cannabinoid system has a significant role in the regulation of immunity which confer them potential therapeutic usefulness in immune disorders and malignancies [reviewed in 46-48].

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This review will focus on the growing body of evidence that exogenous and endogenous cannabinoids activate numerous signal transduction pathways some of which may explain the biologic effects exerted by these substances. Cannabinoid receptor-independent events are also discussed. Readers may found other recent reviews in 45 and 49.

2. SIGNAL TRANSDUCTION PATHWAYS ACTIVATED BY CANNABINOID RECEPTORS

Cannabinoid receptors belong to the large superfamily of heptahelical receptors also named G protein-coupled receptors (GPCRs), that are single polypeptides with seven α-helices-domains traversing the cell membrane seven times, an intracellular C-terminus and an extracellular glycosylated N-terminus. They exert their activity by coupling to effectors through adaptor proteins. Although the classical pathway of signalling involves agonist-promoted binding of the receptor to the heterotrimeric guanosine triphosphate-binding protein (G protein), current evidences suggests that heptahelichoidal receptors may be regulated by interaction with multiple proteins [50]. This is also the case for cannabinoid receptors, although due to their recent discovery, transduction pathways and adaptor coupling are less documented.

2.1. Coupling to G Proteins

Activation of cannabinoid receptors promotes its interaction with G proteins, resulting in guanosine diphosphate/guanosine triphosphate exchange and subsequent dissociation of the α and βγ subunits. These subunits regulate the activity of multiple effector proteins including adenylyl cyclases, ion channels, phosphoinositide 3-kinase, and phospholipases.

Both CB_1 and CB_2 cannabinoid receptors are linked to pertussis toxin-sensitive $G_{i/o}$ proteins. However, the affinity of CB_1 and CB_2 for G_i or G_o proteins may be different as revealed by several studies on cannabinoid ligand binding [51] or regulation of [^{35}S] GTPγS binding [52]. Whereas, activation of both of them display a high affinity for $G_{i,}$ agonist stimulation of CB_1 also result in a high-affinity saturable interaction with G_o but CB_2 receptor do not interact efficiently with G_o. It has been reported that the affinity of CB_1 receptor for G_o is ten fold higher than that of the CB_2 [53].

The agonist-selective G protein signaling by cannabinoid receptors have been extensively studied. Regarding CB_1 receptors, the two endocannabiniods, anandamide and 2-AG, exhibit differential sensitivity for the activation of G_i and G_o. 2- AG produced a maximal stimulation of both G_i and G_o while anandamide was a full agonist in the activation of G_i, but only produced partial activation of G_o. Therefore, these endogenous agonist might divergently activate G-proteins thereby producing different effects [49, 53].

About the exogenous agonist, it has been also reported that HU-210 and WIN55,212 elicited maximal stimulation of $G_{,}$ whereas Δ^9-Tetrahydrocannabinol caused only partial G_i activation. In contrast, only HU-210 induced maximal stimulation of G_o, followed by WIN55,212 and Δ^9-Tetrahydrocannabinol [53, 54].

In most cells, the CB_1 receptor remains precoupled with G proteins even in the absence of an exogenously added agonist [55]. This phenomenon differs from what has been observed for many other GPCR-G protein interactions and may contribute to the constitutively active behaviour attributed to the cannabinoid receptors [56, 57].

Recently, the structural requirements for the receptor-G protein interaction have been extensively studied. It has been described that the juxtamembrane C-terminal region of CB_1 receptor (amino acids 401-417) and the second and third intracellular loops are critical for $G_{i/o}$ protein coupling and that the distal C-terminal tail domain profoundly modulates both the magnitude and kinetics of signal transduction [58]. The CB_1 receptor-$G\alpha_{i3}$ and -$G\alpha_o$ interactions can be competitively disrupted by the C-terminal juxtamembrane peptide, however the receptor- $G\alpha_{i1}$ and -$G\alpha_{i2}$ interactions can be competitively disrupted by third intracellular loop peptides. It can be concluded from these studies that the juxtamembrane C-terminal domain recognizes and regulates $G\alpha_{i3}$ and $G\alpha_o$, whereas the third intracellular loop domain represents the $G\alpha_{i1}$ and $G\alpha_{i2}$ interacting domain of the receptor [55, 59]. Also for interacting with $G_{\alpha16}$, sequences of the second and third intracellular loops and the carboxy-terminus are required [60].

In addition to the linkage to $G_{i/o}$ proteins, coupling of CB_1 to pertussis toxin-insensitive G_s proteins appears to be also possible [61, 62].

Regarding interaction of CB_2 with G_i proteins, it has been shown that anandamide is a partial agonist in the activation of these proteins, whereas 2-AG was proved to be fully efficacious [53, 63]. Regarding exogenous cannabinoids, HU-210 was the only compound that showed maximal activation. In contrast, WIN55,212 and Δ^9-Tetrahydrocannabinol produced submaximal levels of G_i protein activation [53].

About the structural requirements for CB_2-G protein interaction, it has been described that the C-terminal juxtamembrane peptide from the CB_2 receptor failed to compete for $G\alpha_o$ or $G\alpha_i$ as it did in the CB_1 receptor [55, 59]. But it has been described the existence of two cysteins, C313 and C320, that are located in this C-terminal region, that may play important roles for receptor- G protein coupling and receptor desensitization [64, 65]. Also, the third transmembrane domain in the CB_2, particularly the Asp-Arg-Tyr motif, may be crucial for interacting with G proteins because mutations of highly conserved aspartate residues in the second transmembrane domain receptors have also been described to disrupts G-protein coupling [66].

2.2. G Protein-Independent Signal Transduction

An increasing number of observations point to different mechanisms through which cannabinoid receptors can initiate intracellular signals without G proteins participation. Cannabinoids has been shown to induce sphingomyelin hydrolysis independently of G proteins and through interaction with the adapter protein FAN (factor associated with neutral SMase activation) [67]. FAN protein was initially described as a p55 TNF receptor-associated protein that was essential for the activation of neutral sphingomyelinase. Interaction of rat CB_1 cannabinoid receptor expressed in astrocytes was demonstrated by coimmunoprecipitation experiments showing that the binding of FAN to the CB_1 receptor was enhanced by THC

and prevented by SR 141716 [67]. Coupling to sphingomyelin hydrolysis through Fan protein was evidenced by the fact that cells expressing a dominant-negative from of FAN were refractory to THC-induced sphingomyelin breakdown [67]. The activation of the cannabinoid receptor CB_1 causes, by this pathway, sphingomyelin hydrolysis that may mediate metabolic processes [68-70].

Other non-G proteins that could mediate cannabinoid receptor signalling are arrestins. Arrestins bind to activated 7 TM receptors and attenuate further receptor signalling thereby causing desensitisation (see below). Currently, four mammalian arrestins have been identified, two visual and two non-visual (β-arrestin-1 and –2) being the latter widely distributed. Recent studies reveal that non-visual arrestins regulate several aspects of 7 TM signalling including the extracellular signal regulated kinase (ERK) cascade and the c-jun N-terminal kinase 3 (JNK3) cascade [71, 72]. Although there are some evidences that cannabinoid receptors interact with β-arrestins (see 2.3. Receptor desensitisation), coupling through arrestins to downstream signalling mechanisms has not been reported.

Although this mechanisms were initially observed with the CB_1 cannabinoid receptor, similar pathways could operate with the CB_2 receptor.

2.3. Receptor Desensitisation

Like other seven transmembrane (7 TM) domain receptors, CB_1 receptor is also regulated by desensitisation and internalisation after agonist activation. Usually, GPCRs desensitisation is primarily regulated by phosphorylation by Ser/Thr kinases and by interaction with arrestins [72, 73]. Arrestins are versatile adapter proteins that form complexes with GCPRs mediating the desensibilization process. Desensitization of CB_1 cannabinoid receptors after chronic treatment with cannabinoid agonists has been shown either *in vivo* [74-78], as in culture cells [79] or in expression systems [80, 81]. The mechanism of receptor desensitisation is not well understood, but recent data indicate that desensitisation of cannabinoid receptors is mediated by β-arrestin following the prototypical pathway for GPCRs [79, 82]. Other mechanisms of receptor desensitisation may involve phosphorylation by second messenger-dependent kinases. Garcia and co-workers demonstrated that phosphorylation of CB_1 by PKC, disrupted its modulation of ion channels. Direct phosphorylation of rat CB_1 in a Ser residue by PKC was demonstrated by using a fusion protein incorporating the third intracellular loop of CB_1 [83]. This phosphorylation may mediate a generalized cellular hypore-sponsiveness, thus causing heterologous desensitization.

Desensitization of CB_2 has been less studied but experiments performed in CHO cells stably transfected with CB_2 receptor sowed that phosphorylation at serine 352 is previous to receptor internalisation [84]. Treatment with the cannabinoid agonist CP 55,940 increased ser 352 phosphorylation that was maintained for 8 hours [84].

2.4. Downstream Effector Systems

2.4.1. Adenylyl Cyclase

One of the most extensively studied properties of cannabinoid receptors is their ability to couple to pertussis toxin sensitive G-protein ($G_{i/o}$) to inhibit adenylyl cyclase in cells with naturally expressing CB_1 receptors or transfected with both cannabinoid receptors [85]. It is noteworthy that cells expressing CB_2 receptors naturally are relatively insensitive to cannabinoid-induced inhibition of cAMP production [86].

Inhibition of adenylyl cyclase has been characterized in brain tissue [87-89] and different cells lines like mouse N18TG2 neuroblastoma [90, 91], COS-M6 [92], CHO cells [85, 63] and prostate PC-3 cells [27].

At CB_1 receptor both anandamide and 2AG appear to be full (or nearby full) agonist in the inhibition of adenylyl cyclase [53, 85, 93]. However *via* the CB_2 receptor, 2- AG but not anandamide was recently demonstrated to be a full agonist in the inhibition of cAMP accumulation [63, 93].

In addition to inhibition cAMP accumulation, CB_1 but not CB_2 receptors can stimulate cAMP formation under certain conditions, consistent with a putative G_s linkage of this receptor [61]. Stimulation of adenylyl cyclase has been reported in pertussis toxin-treated cells, suggesting that in the absence of functional $G_{i/o}$ coupling, the CB_1 receptor can activate G_s [94]. The isoform of the adenylyl cyclase expressed in cells is predicted to be the most important determinant of the activation or inhibition of the enzyme. So, it has been observed activation of the cannabinoid receptor CB_1 and CB_2 inhibit the activity of adenylyl cyclase types I, V, VI and VIII whereas types II, IV and VII are stimulated by receptor activation. The inhibition of type III by cannabinoids is observed only when forskolin is used as stimulant. About the activity of adenylyl cyclase type IX, it is inhibited only marginally by cannabinoids [95].

Modulation of the intracellular cAMP concentration, and thereby regulating for example, the phosphorylation of substrate proteins by protein kinase A, can result in changes in gene regulation and cellular activity [96, 97].

2.4.2. Ion Channels

2.4.2.1. Inhibition of High-Voltage-Gated N, L and P/Q Ca^{2+} Channels

Cannabinoid agonists reduce the amplitude of voltage-gated calcium currents in neuronal cells through $G_{i/o}$ proteins. Inhibition of N-type voltage-gated channels by cannabinoids has been demonstrated in several neural cells using whole-cell voltage clamp technique and intracellular calcium measurement with the dye Fura-2 [98-102]. Inhibition of N-Ca^{2+} currents were blocked by CB_1 antagonists and by treatment with pertussis toxin showing the participation of cannabinoid receptor and $G_{i/o}$ proteins in the mechanism of channel inhibition [98-102].

The CB_1 receptor also inhibits L-type voltage-gated channel in arterial smooth muscle cells through a pertussis toxin-sensitive pathway [103]. However, in the neuroblastoma/glioma hybrid cell line NG108-15, the cannabinoid agonist desacetyllevonantradol (DALN) induced a stimulatory effect on L-type calcium channels that was abolished by SR 141716 but resistant to pertussis toxin treatment [104] although completely blocked by introducing anti-G_s antibodies into the cells [105]. This results together with the observation of an elevation in cAMP induced by DALN in neuroblastoma cells, suggest a coupling of the

cannabinoid receptor with G_s proteins. The stimulatory effect of DALN on Ca^{++} uptake was mediated by cAMP and PKA and modulated by PKC and calmodulin [104, 105].

Cannabinoid agonists inhibit P/Q-type calcium channels in hippocampal neurones expressing CB_1 [106] and in neurones transfected with the cannabinoid receptor [107].In both cases, inhibition was reverted by pertussis toxin and the CB_1 antagonist SR 141716.

Because the channels that underlie this currents are mainly located presynaptically and are required for evoked neurotransmitter release, modulation of calcium channels by cannabinoid receptor may be one mechanism by which cannabinoids may decrease transmitter release from presynaptic neurons [108].

2.4.2.2. Modulation of Potassium Channels

Inward-rectifier potassium (K_{ir}) channels comprise a superfamily of K^+ channels responsible for setting the resting membrane potential, controlling the excitation threshold and secreting K^+ ions. Inward rectification is due to a voltage-dependent block of the channel by intracellular modulators [109]. This superfamily of channels is divided into seven subfamilies and uniquely the members of the $K_{ir}3$ subfamily are activated by G proteins upon stimulation of GPCRs and are also named G-protein-gated inwardly rectifying K^+ channels (GIRK). Activation of this GIRK channels by cannabinoids has been studied specially in *Xenopus laevis* oocytes coexpressing the CB_1 receptor and GIRK1[110] or GIRK1 plus GIRK4 [111, 112]. Co-expression of CB_1/GIRK1/GIRK4 and different G protein subunits revealed that modulation of GIRK by cannabinoid receptor was mediated by $\beta\gamma$ dimmer and not by G_α [112]. Activation of K_{ir} channels reduces the rectification of inward potential thus regulating the excitability of neurons which may explain some of the physiological effects of cannabinoids. Recently, coupling of cannabinoid receptors to GIRK channels *in vivo*, has been suggested using GIRK-null mutant mice [113]. In this mice, analgesic effect of the cannabinoid agonist WIN 55,212, was eliminated suggesting an important role for the modulation of GIRK channels by cannabinoids in the antinociceptive effects of this compound [113].

Cannabinoids also modulate other voltage-gated potassium currents in hippocampal neurons including the rapid-inactivating potassium A (I_A) current [114, 115] and I_M currents [115]. However, modulation of I_A current by cannabinoids involves a complex pathway through activation of PKA by cAMP and channel phosphorylation [117, 118].

2.4.2.3. Inhibition of Sodium Current

Depression of inward sodium current by THC was demonstrated in neuroblastoma cells using the whole-cell voltage-clamp technique. THC decreased the peak amplitude and increased both the time to peak and tau for recovery, showing an additional mechanism for cannabinoids in the depression of action potentials [119, 120].

CB_2 channels seem to be independent of channel activation. However, in *Xenopus laevis* oocytes co-expressing CB_2 and GIRK channels, coupling of CB_2 to GIRK was observed [112].

2.4.3. MAP Kinase

One of the most interesting research areas is the regulation of cellular growth by cannabinoids. The endogenous cannabinoid anandamide and other cannabinoid agonists regulate cellular proliferation in many cell types but the underlying biochemical mechanisms remain unclear. Although regulation of cellular growth have been usually associated with tyrosine kinase receptors, recent evidences show that GPCRs can stimulate the mitogen-activated protein kinase (MAPK) cascade and thereby induce cellular proliferation. The mammalian MAPK family consists of three subfamilies with multiple members: the extracellular signal-regulated kinases (ERK), the Jun amino-terminal kinases/stress-activated kinases (JNK/SAPK), and the p38 MAPKs. While ERK is involved in regulation of cell division and growth, the other two subfamilies are activated by stress signals and inflammatory cytokines and have been related with cellular death and immune disorders [121]. Each MAPK cascade is organized in a three member-protein kinase tandem in which the MAPK is activated by dual phosphorylation in both tyr and ser/thr residues by a dual MAPK kinase (MEK) which in turn is activated by phosphorylation by a MAPK kinase kinase (MEKK).

Since the first observation of activation of MAP kinase pathway by anandamide [122], extensive molecular and pharmacological studies have demonstrated that cannabinoid receptors activate the extracellular signal-regulated kinase cascade both *in vivo* and *in vitro*. Using CHO cells expressing human CB_1, the group of Dr. Casellas demonstrated that cannabinoid treatment induced phosphorylation and activation of the two isoforms p42 and p44 kDa of ERK by a pathway that involved CB_1 receptor and $G_{i/o}$ proteins but independent of cAMP [123]. In cortical astrocytes and glioma C6 cells, THC and HU-210 induced glucose metabolism through stimulation of ERK cascade [68, 124]. Stimulation of ERK kinase by THC and methanandamide has been recently shown in prostate epithelial cells [125]. THC promoted Raf-1 translocation to the membrane and phosphorylation of the subsequent cascade members in both astrocytes and prostate cells [68, 125]. While G proteins are involved in the activation of MAP kinase pathway by cannabinoids, the pathway seems to be independent of cAMP signalling [68]. Efforts then, have been made to reveal the pathway that link the activation of MAP kinase and cannabinoid receptors.

Regarding activation of the MAP cascade, there are controversial results showing an inhibition of ERK by cannabinoids under certain conditions. In primary mouse splenocytes, cannabinoid compounds inhibited AP-1 transcription factor activity by decreasing ERK activity [126]. Inhibition by anandamide of NGF-induced sustained ERK activation was also observed in PC-12 cells transfected with CB_1 receptor [127]. Differences observed may depend on the cell type, specially in the Raf-1 isoforms expressed in the cell that are differently activated by cAMP signalling. There are three members of Raf family, A-Raf, B-Raf and C-Raf or Raf-1 that are differently expressed in the cells and differently regulated by cAMP [128]. The three can activate MEK resulting in activation of ERK. cAMP activates Rap1, a small G protein, which can activate B-Raf but not Raf-1. By contrast, cAMP leads to inhibition of Raf-1 which is

ubiquitous [reviewed in 129]. As cannabinoids decrease cAMP content, the general response of cannabinoids is activation of ERK cascade. In cells where B-Raf is expressed, such as neuronal or hematopoietic cells, cannabinoids can inhibit ERK cascade.

The CB_2 cannabinoid receptor was found to be coupled to ERK cascade activation and expression of the growth-related gene Krox-24 in transfected CHO cells [130]. Increase in ERK activity in response to cannabinoid agonists, has been also observed in splenocytes [131] and hematopoietic cells [132] expressing the CB_2 receptors. A phosphorylation increase in the two isoforms of ERK (p42 and p44) was observed in HL-60 cells treated with 2-AG [133,134], that was blocked by pertussis toxin treatment, indicating the requirement for $G_{i/o}$ proteins [133].

There are other many studies showing the involvement of the ERK kinase pathways in the cell growth-regulatory properties of cannabinoids, inferred by the blocking actions of different inhibitors that have been omitted for the lack of space.

Several studies presented evidence that cannabinoid agonists activate stress-regulated MAP kinases. Studies in Chinese hamster ovary cells stably transfected with CB_1 evidence that THC and the endogenous cannabinoids anandamide and 2-AG induced the activation of JNK and p38 MAPK [139]. Activation of JNK and p38 kinases has also been demonstrated in vascular endothelial cells naturally expressing CB_1 [136]. However, in hippocampal slices, THC and 2-AG activated p38 but not JNK [137].

Differences in results may be due to the complex regulation of MAP kinase cascades by GPCRs and the spatio-temporal control in MAP kinase signalling. It is important to point out the risk of misinterpreting experiments that show activation or inhibition of MAPK at selected times because in some cases, the effect of some agonists is to delay the activation or inhibition of MAPK without modifying activity.

2.4.4. Mechanisms that may Link Cannabinoid Receptors with MAPK Cascade

2.4.4.1. Tyrosine Kinase Receptors Transactivation

Several mechanisms have been proposed for the coupling of metabotropic receptors to MAP kinase cascade. They include (1) signals initiated by classical G protein effectors, e.g. PKA, PKC, (2) signals triggered by direct interaction between β-arrestins and components of the MAPK cascade, (3) signals initiated by cross-talk between GPCRs and receptor tyrosine kinases (RTKs) by a mechanism named "receptor transactivation", (4) signals regulated by βγ subunits upon dissociation from heterotrimeric G proteins, and (5) signals initiated by cAMP independently of PKA. Regarding cannabinoid receptors, only the third and the fourth points have been investigated. The best studied mechanism for cross-talk between TRKs receptors and GPCRs is the transactivation of the EGF receptor [reviewed in 137]. The activation of GPCRs can lead to ligand-independent phosphorylation of key tyrosine residues in TRKs, which creates docking sites for proteins that contain phosphotyrosine binding domains, triggering the consequent signalling cascade. In a recent study performed by Galve-Roperh *et al*. in the astrocytoma cell line U373 MG, the authors showed that whereas experiments conducted with pharmacological inhibitors suggested the participation of certain mechanisms for CB_1-mediated ERK activation, detailed study indicate that EGF receptor transactivation was not involved [138]. Moreover, cannabinoid agonists inhibited EGF receptor activation in skin tumors *in vivo* [35].

2.4.4.2. PI3K Activation

Phosphoinositide-3 kinase (PI3K) comprises a family of dual kinases that phosphorylate the 3'-OH position of the inositol ring of phosphatidylinositol and other phosphoinostides (PI), generating 3'-PIs[139]. Those 3'-PIs facilitate the recruitment of pleckstrin homology (PH) domains-containing proteins to the membrane which is crucial for their activation. One of the most interesting proteins of this pathway is protein kinase B (PKB) which is involved in regulation of anti-apoptotic signals [140]. Additionally, PI3K has a protein kinase activity which might be involved in PI3K signalling. Phosphoinositide-3 kinase isoforms may be also required to link GPCRs to MAPK. PI3K isoform I_B, has been characterized as a direct target of βγ complexes dissociated from G_i [141].

Recent studies have emphasized the role of PI3K pathway in the MAPK activation exerted by cannabinoids. THC induced activation of PKB in CHO cells stably transfected with the CB_1 [142]. This effect was mimicked by anandamide and other cannabinoid agonists as HU-210 and CP 55,940 and was prevented by PI3K inhibitors, indicating the involvement of PI3K activation [142]. It has been recently shown that CB_1 receptor coupling to Erk activation in U373 MG human astrocytoma cells, depended on G_i protein dissociation and subsequent PI3K activation [138]. The functional relevance of PI3K-dependent ERK activation was confirmed by assessing the anti-apoptotic action of cannabinoids in astrocytoma cells that was abrogated by the PI3K inhibitors wortmannin and LY 294,002 [138]. In prostate PC-3 cells, induction of NGF synthesis by cannabinoids was dependent on ERK pathway and PI3K activation. The activation of ERK was by a PI3K-dependent mechanisms inasmuch as it was blocked by the PI3K inhibitor LY 294,002 [125]. In rat oligodendrocytes, cannabinoid agonists exerted a pro-survival action through activation of the PI3K/PKB pathway. This effect was reverted by pertussis toxin and by cannabinoid receptor antagonists [143]. Both CB_1 and CB_2 seemed to be involved in the protective action of cannabinoids since only SR141716 and SR 144528 in combination inhibited the effect, and both receptors are expressed in these cells [143].

2.4.4.3. FAK Activation

The focal adhesion kinase (FAK) is a cytoplasmic tyrosine kinase that localizes to the regions of the cell that attach to the extracellular matrix. It functions as component of the integrin signalling pathway, transmitting signals from the extracellular matrix into the cytoplasm that control cell motility [144]. There are a number of biochemical pathways that can be activated by FAK including MAP kinase cascades. When FAK is activated, it became phosphorylated in several tyrosine residues transforming in binding sites for adaptor proteins, causing its recruitment and activation of

SOS/Ras/MAPK pathways. In cells expressing B-Raf, FAK signalling leads to the activation of Rap1/B-Raf/MEK/MAPK pathway in a sustained fashion [145]. In addition, FAK might play a role in the activation of the JNK kinase pathway and p38 kinase pathways [146, 147]. Anandamide, CP 55940 and WIN 55,212 increased phosphorylation of $FAK^+6,7$, a neural isoform of FAK, in hippocampal slices and in cultured neurons [148]. The effect could be blocked by the CB_1 antagonist SR 141716 and pertussis toxin, suggesting the involvement of the CB_1 receptor and $G_{i/o}$ protein [148]. A more detailed study of the mechanism involved in cannabinoid activation of neural FAK, revealed that 2-AG and THC increased phosphorylation in Tyr-397 residue, which is crucial for FAK activation [149]. Cannabinoids also increased phosphorylation of p130-Cas, a protein associated with FAK, but were inactive on PYK2, a tyrosine kinase related to FAK [149]. Those effects were mediated by cannabinoid-induced decrease in cAMP [148, 149]. Because in hippocampal slices from Fyn-/- mice the effects of cannabinoids on $FAK^+6,7$ phosphorylation were abolished, it was concluded that in the pathway activated by endocannabinoids the association between Fyn and $FAK^+6,7$ play an important role [149]. Different results were obtained in mouse neuroblastoma N1E-115 cells in which HU-210 was found to induce tyrosine phosphorylation of focal adhesion kinase-related non-kinase (FRNK) but not FAK [150]. HU-210-induced tyrosine phosphorylation of FRNK was mediated by CB_1 and by inhibition of the cAMP pathway [150]. This mechanisms might play a role on endocannabinoid-induced modulation of synaptic plasticity, cell migration and neurite remodelling and indicate new mechanisms for cannabinoid regulation of MAP kinase cascade.

2.4.5. Intracellular Calcium and PLC Activation

Different cannabinoid agonists have been shown to induce a rapid and transient elevation of intracellular Ca^{2+}, measured using conventional fluorescence spectrometry of the calcium binding dye Fura-2. Either the endogenous cannabinoid 2-Arachidonoylglycerol and or the exogenous cannabinoids THC and WIN 55212-2 at nanomolar concentrations, induced a rapid intracellular free Ca^{2+} concentration rise in neuroblastoma NG108-15 cells that was abolished by pre-treatment of cells with the antagonist SR 141716, suggesting the involvement of CB_1 receptor [151-153]. The effect induced by 2-AG was inhibited by pre-treatment with the PI-phospholipase C (PLC) inhibitor U73122, pointing to a PLC activation by cannabinoid receptor with the consequent increase of IP_3 and release of Ca^{2+} from intracellular stores. Additional evidence for the activation of PLC by cannabinoid agonists comes from the studies by Netzeband *et al*. showing that the PLC inhibitor U-73122 and the IP_3 receptor antagonist xetospongin C, blocked the enhancement of intracellular calcium concentration produced by HU-210, WIN 55212-2 or methanandamide in cerebellar granule neurones [154]. However, in hippocampal neurones, micromolar doses of THC induced a delayed increase in intracellular calcium that was dependent on extracellular calcium and was inhibited by SR 141716 and lanthanum, a potent Ca^{2+} channel blocker, suggesting that a plasma membrane channel was activated by the cannabinoid receptor [155]. Anandamide also induced an intracellular Ca^{2+} concentration rise in human-derived umbilical endothelial cells but this effect was not mediated by cannabinoid CB_1 receptor [156]. Similarly, a CB_1/CB_2 independent mechanisms caused an increase in intracellular calcium concentration induced by micromolar doses of CP55,940 in canine kidney cells [157] and in bladder cancer cells [158]. The cannabinoid agonist induced a $[Ca^{2+}]_i$ rise by releasing Ca^{2+} from intracellular thapsigargin-sensitive calcium stores independently of PI-phospholipase C activation [157,158].

In an attempt to determine the signalling pathway between CB_1 receptor and Ca^{2+} mobilisation, the group of Dr. Molleman studied the effect of CP55,940 on membrane currents by whole cell patch clamp technique. CP55,940 induced a transient outward current in cells derived from vas deferens carcinoma that was prevented by pertussis toxin and SR 141716. CP55,940 induced an intracellular concentration calcium rise released from thapsigargin-sensitive intracellular stores that was dependent on extracellular calcium entrance and was independent of PLC activation suggesting that there is a mechanisms of capacitive Ca^{2+} entry into intracellular stores in vas deferens derived cells [159].

2.4.6. Arachidonic Acid Metabolism

There are many data that correlate cannabinoids with arachidonic acid metabolism. Anandamide and exogenous cannabinoids induce arachidonic acid mobilization and activation of the enzymes of arachidonic acid cascade in many cells. It is important to note that anandamide degradation into cells by fatty acid amide hydrolase (FAAH) yield arachidonic acid that could mediate some biological actions of endocannabinoids like vasorelaxation but, in this case, the effect is independent of cannabinoid receptors [160]. Independently, activation of cannabinoid receptors may induce generation of arachidonic acid and may also modulate the enzymes of the arachidonic acid cascade. Three enzymes are involved in the release of arachidonic acid from arachidonate derivatives present in the membrane phospholipid domain: phospholipase D, phospholipase C and phospholipase A_2. The three enzymes have been shown to be activated by cannabinoids [161-164]. Activation of PLD by cannabinoids depends on cell type. THC stimulated PLD in mouse peritoneal cells [161] and in human platelets [162] while it failed to activate PLD in CHO cells expressing CB_1 [161]. Anandamide activated PLD in PC12 cells but not in NIH 3T3 fibroblasts [163]. Phospholipid hydrolysis by PLD results in arachidonate containing diacylglicerols generation that, after hydrolysis by DAG lipases, may release arachidonic acid. However activation of PLA_2 is the primary pathway through which arachidonic acid is liberated from phospholipids. The main mechanism of cytosolic PLA_2 activation is phosphorylation by MAP kinase although the participation of intracellular calcium is also necessary for fully activation of the enzyme. THC, cannabidiol induced activation of PLA_2 in platelets, peritoneal cells and synaptosomes [161, 162, 164]. As MAP kinase activation seems to be a general transduction mechanism of cannabinoid receptors, and cannabinoids induce intracellular calcium mobilization in some conditions, activation of PLA_2 by cannabinoids could be a general signalling pathway although it remains unexplored in many cells. Intracellular arachidonic acid release and its

metabolites has been shown to be induced by cannabinoids in several cells [155, 165, 166,] and *in vivo* [167]. In hippocampal neurones, THC induced a release of arachidonic acid that was blocked by the CB1 receptor antagonist SR 141716, but not by pre-treatment with pertussis toxin, suggesting an involvement of the cannabinoid receptor by a mechanism independent of G_i coupling [155]. However, in PC12 cells release of arachidonic acid by anandamide seems to be a cannabinoid receptor-independent phenomena [164].

When formed, arachidonic acid is metabolised by three main pathways. It can be converted into prostaglandins, prostacyclins and thromboxanes by the bi-functional enzyme cyclooxygenase (COX). COX exists in two isoforms commonly known as COX-1 and COX-2 that differ in their tissue distribution and regulation. COX-1 is constitutively expressed in the majority of cells and mediate vascular homeostasis and other basal functions as water reabsorption or gastric acid secretion whereas COX-2 is an inducible and pro-inflammatory enzyme [169]. Arachidonic acid may also be transformed by lipooxigenases (LO) that yield hydroperoxyeicosatetraenoic acids (HPTE) which may be converted in leukotrienes, hepoxillins, trioxillins and lipoxins. And finally, arachidonic acid can be metabolized by cytochrome p450 enzymes to 5,6-, 8,9-, 11,12-, and 14,15-epoxyeicosatrienoic acids (EETs), their corresponding dihydroxyeicosa-trienoic acids (DHETs), and 20-hydroxyeicosatetraenoic acid (20-HETE). It has been recently shown that cannabinoids may modulate arachidonate metabolic enzymes. In human platelets THC inhibited COX-2 activity blocking the synthesis of its pro-inflamatory metabolites with the redistribution of products towards lipoxygenase pathway [170]. However, in human neuroglioma cells THC and methanandamide stimulated COX-2 mRNA expression and subsequent PGE(2) synthesis *via* a non cannabinoid receptor-mediated mechanism [171].

By other hand, endocannabinoids anandamide and 2-AG may be metabolised by arachidonic acid enzymes as effectively as arachidonic acid with the subsequent generation of lipid mediators [172-174].

2.4.7. Nitric Oxide

Nitric oxide (NO) is a short lived free radical and ubiquitous cell-signalling molecule produced by several tissues, including endothelial cells, neurons, astroglial cells and macrophages. It is involved in numerous cellular functions such as controlling vascular homeostasis [175] and synaptic transmission [176]. NO production can also be induced by proinflammatory factors under pathological conditions [177].

In living cells, NO is synthesized from L-arginine *via* the catalytic action of the enzyme NO synthase (NOS). Three types of enzymes have been identified and characterized. Two of the three are constitutive and expressed in specific cells types (NOS I or neuronal and NOS III or endothelial), whereas the expression of the third isoform (NOS II or inducible) can be induced by cytokines. The constitutive isoform (cNOS) is a calcium-calmodulin-dependent enzyme [178] whereas the inducible isoform (iNOS) is a calcium-independent enzyme and it binds calmodulin tightly [179].

Cannabinoids have been reported to interact with cNOS and iNOS isoforms. NO production is stimulated by anandamide in human monocytes [180], rat median eminence fragments [181], human saphenous vein segments [182], cultured human arterial endothelial cells [183, 184], cultured human umbilical vein endothelial cells [185] and leech or muscle ganglia [186]. This stimulation could explain some cannabinoids effects like vasodilatation and neurotransmission release inhibition because it has been described that both anandamide and the NO-generating agent *S*-nitroso-*N*-acetyl-penicillamine could inhibit the release of preloaded dopamine from invertebrate ganglia [187]. The antagonist of these responses by the substrate competitor N^G-nitro-L-arginine methyl ester (L-NAME) confirms that cNOS was involved in the generation of NO [181]. The stimulation of NO synthesis was blocked by SR141716, implicating the mediation of CB_1 receptor. In some studies, it has been observed that an increase in intracellular Ca^{2+} concentration is required for NO generation consistent with the stimulation of a Ca^{2+}-regulated constitutive isoform of NOS [183, 184]. Also, anandamide uptake by human endothelial cells was required by NO production [185].

Cannabinoids produced an inhibition of iNOS transcription and NO production in response to lipopolysaccharide plus interferon-γ in different tissues [188]. This effect has been described, for example, in saphenous vein endothelium [182], neonatal mouse astrocytes [189] and microglial cells [190].The CB_1 receptor was implied in these inhibitions since this response was reversed by SR141516. Due to these effects, cannabinoids could be used as therapeutic agents in NO-mediated inflammation leading to neurodegeneration [191, 192, 193]. The modulation of iNOS induction by cannabinoids required NO production. It has been suggested that the mechanism for suppression of iNOS induction involved the inhibition of adenylyl cyclase isoforms 5 and 6 by NO [182, 194].

About CB_2 receptor, it has been reported the attenuation by Δ^9-THC of iNOS induction in RAW 264.7 cells and also a mechanism involving a decrement in cAMP levels was implied [188].

The activations of both cannabinoid receptors were implied in the inhibition of IFN-γ /bacterial lipopolysaccharide (LPS)- iNOS production of bone marrow derived feline macrophages because the inhibitions were reversed by the antagonists SR141716 and SR144528. Thus by inhibiting NO production in these cells, cannabinoid are expected to reduce host resistance to several infections [195]. So, for example, epidemiological data suggest that HIV positive marihuana smokers progress to symptomatic AIDS more rapidly than those who do not smoke it [196].

It have been proposed that there could exist signalling convergences among endocannabinoid, morphine, eicosanoid systems and NO production [197]. So, morphine receptor μ3 and cannabinoid receptor CB_1 activation, leads to intracelullar calcium levels increase followed by the cNOS activation. cNO can prevent tissue damage caused by iNOS because it induces the down-regulation of iNOS expression through cAMP and NFkB inhibition. Nevertheless, direct proinflammatory cytokine action can stimulate iNOS, eliminating the other cascades and leading to a continuous production of NO for hours and days. These excessive levels of NO are able to produce tissue and cellular injury [197].

2.4.8. De Novo Synthesis of Ceramide

The Dr. M. Guzman's group accomplished an important breakthrough in understanding the regulation of ceramide levels by cannabinoid receptor ligands. THC induced a sustained increase in ceramide levels that was involved in the apoptotic effect caused by cannabinoids in rat glioma C6 cells [70, 198]. The origin of the ceramide responsible for the cannabinoid-induced apoptosis was studied by pharmacological inhibition of the synthesis pathway and testing enzymatic activity. Inhibition of both serine palmitoyltransferase with L-cycloserine and ceramide synthase with fumonisin B_1, prevented the THC-induced death of glioma cells [199]. By other hand THC enhanced serine palmitoyltransferase activity, which catalyses the rate-limiting step in ceramide synthesis, suggesting that the ceramide accumulated upon THC treatment is *de novo* synthesized [199].

2.5. Transcription Factors

Transcription factors are proteins that control the expression of genes and therefore they are the effectors of the signalling cascades that regulate cell development and functioning. They can be divided in two categories: the constitutive transcription factors and the inducible transcription factors [200]. The former are constitutively expressed in the cell, are usually already bound to the DNA and are activated by phosphorylation or/and interaction with other factors and proteins, whereas the expression of the latter are controlled by constitutive transcription factors and induced rapidly upon cell stimulation. Because of their rapid induction, these genes have been termed "immediate-early genes" [200]. One of the best studied transcription factor activated by cannabinoid agonists is c-Fos. c-Fos and c-Jun proteins are AP-1 transcription factors induced and activated following many stimuli. Activation of c-Fos has been extensively investigated *in vitro* and *in vivo*, and is frequently used to study the responsiveness to cannabinoids as a marker of cellular activation [201-203]. THC and anandamide exogenously administrated increased the expression of c-Fos in different areas of the rat brain [203-205]. The effect of THC on c-Fos induction in the nucleus accumbens was prevented by the CB_1 antagonist SR 141716 [205]. In hippocampal neurones of living mice, THC induced the expression of c-Fos protein and other immediate-early genes by a mechanism dependent on Map kinase kinase activation [206]. Regulation of c-Fos and c-Jun in the periphery seems to be different as expression of c-Fos in the vagus and the nucleus of the solitary tract induced by emetic agents in the ferret, was reduced by THC acting through CB_1 receptors [207]. In a rat model of inflammation, the cannabinoid agonist WIN 55,212-2 also suppressed c-Fos expression in the spinal cord by a CB_1- and CB_2-dependent mechanism [208] and in mouse splenocytes cannabinol decreased nuclear expression of c-Fos and c-Jun and inhibited binding to AP-1 sites of the interleukin-2 promoter, suggesting a mechanism for cannabinol-induced immunosuppression [209].

Other transcription factor that has been shown to be activated by cannabinoid agonists is Krox-24. The Krox family of immediate-early gene proteins are of particular interest because they may be involved in neuronal plasticity and hippocampal long-term potentiation. Activation of Krox-24 expression has been demonstrated in human astrocytoma cells treated with CP 55940 [210]. The specific involvement of cannabinoid receptors in Krox-24 induction was demonstrated in CHO cells transfected with the human CB_1 receptor [123] and with the human CB_2 receptor [130]. A similar coupling of CB_2 to the regulation of Krox-24 was also observed in human promyelocytic cells [130]. Induction of Krox-24 by the cannabinoid agonist CP 55940 also occurs *in vivo* [211].

A potential upstream mediator of Krox induction is cyclic AMP-responsive element binding protein (CREB), a constitutive transcription factor that has been implicated in numerous memory paradigms. CREB is involved in the cAMP signalling cascade that has been shown to be modulated by G-coupled cannabinoid receptors. Inhibition of CREB activation by different stimulus has been reported either in nerve cells or in immune cells. Treatment of hippocampal neurons with WIN 55212-2 protected cells from NMDA-induced neurotoxicity and reversed the up-regulation of CREB produced by NMDA treatment [212]. The modulation of CREB activation may also be a mechanism of cannabinoid-induced immune modulation in immune cells. THC inhibited CREB activation in spleen cells activated with forskolin [213], whereas the CB_2 ligand cannabinol, decreased the activation of CREB in stimulated splenocytes and thymocytes [214-216].

Regarding cannabinoid activation of nuclear transcription factor kappaB (NFκB) data are somewhat controversial. Activation of NFκB by cannabinoid agonists have been demonstrated in the natural killer-like cell line NKB61A2 [217], in HL-60 cell transfected with CB_2 receptor [218] and in PC-12 cells transfected with a reporter gene to measure NFκB activation [219]. However, in activated thymocytes, cannabinol inhibited NFκB activation suggesting a mechanism for cannabinoid-induced immune suppression [215, 216]. Anandamide induced a dose-dependent inhibition of NFκB activated by TNFα treatment of Jurkat cells by a mechanism independent of CB_1 and CB_2 [220]. Anandamide inhibited one of the kinases that phosphorylate the NFκB inhibitory protein (IκB) thereby decreasing IκB degradation [220].

3. RECEPTOR-INDEPENDENT EFFECTS

Some of the effects caused by cannabinoid agents appear to be dissociated from stimulation of cannabinoid receptors. Due to their lipophilic nature, cannabinoids may interact non-specifically with cell membrane components causing membrane perturbations that can induce cellular responses. In some cells, cannabinoid agonists induce an increase in intracellular calcium concentration, which is not altered by cannabinoid antagonists. Felder *et al*. demonstrated more then ten years ago that the synthetic cannabinoid analogue CP55,940 rapidly increased intracellular calcium concentration in fibroblast cells stably transfected with the cannabinoid receptor CB_1 as well as in untransfected cells [221]. Similar properties of cannabinoid agonists were observed in renal tubular cells in which either CP55,940 or olvanil, a presumed cannabinoid and vanilloid receptors modulator, rapidly induced an intracellular calcium

concentration rise that was not blocked by cannabinoid or vanilloid antagonists [222, 223]. The same authors observed intracellular calcium concentration rise in response to CP55,940 in different cell types including bladder cancer cells, human osteosarcoma cells and neuroblastoma cells [224]. In all cases, $[Ca^{2+}]_i$ was measured using the fluorescent dye Fura-2 as an indicator, and the $[Ca^{2+}]_i$ increase was not affected by CB_1 or CB_2 receptors antagonists, indicating that the CP55,940 action was independent of cannabinoid receptor activation [222-224].

Other actions that may be receptor-independent are the immunomodulatory properties of cannabinoids. Several studies have reported that high concentration of cannabinoid ligands induced inhibition of immune cells proliferation and immune functions [reviewed in 225]. Although the majority of these effects were receptor-dependent, some of them were elicited by cannabinoids without receptor participation raising the question of high-concentration non-specific membrane effects [reviewed in 45]. Puffenbarger *et al*. [226] described that either exogenous or endogenous cannabinoids were able to inhibit LPS-inducible cytokine mRNA expression in microglial cells. However, the paired enantiomers CP55,940 and CP56,667, caused the same inhibition than THC or methanandamide. Moreover, neither the CB_1-selective antagonist SR1 nor the CB_2-selective antagonist SR2, were able to reverse the inhibitory effect of cannabinoids, pointing to a receptor-independent mechanism [226]. Similar results were obtained by Facchinetti *et al*. [227] who recently reported that cannabinoids ablate TNFalpha release in LPS-stimulated microglial cells without CB_1 or CB_2 participation. However, authors propose the existence of a new putative cannabinoid receptor which could mediate the effects caused by cannabinoids in these cells [227]. A biphasic action of THC on pro-inflammatory cytokine production by mononuclear cells was also observed by Berdyshev *et al*. [228]. As the authors point, the inversion of the THC inhibitory effect with an increase in concentration suggests the existence of non-specific interactions of this compound with immune cells.

Cannabinoids may also modulate cell proliferation and in some cases this effect has been shown to occur without receptor participation. Different cannabinoid agonists induced human B-cell growth increase at nanomolar concentrations and B-cell growth inhibition at micromolar concentrations [229]. The former effect was blocked by receptor antagonists as well as pertussis toxin whereas the latter was not modified either by cannabinoid antagonists or PTX [229]. Similar biphasic effect of THC was described in prostate malignant cells. Whereas nanomolar doses of THC increased cell proliferation [125] and nerve growth factor induction [230] *via* CB_1 activation, higher doses induced cell apoptosis independently of cannabinoid receptor [231]. In lymphocytes, THC induced apoptosis through the regulation of Bcl-2 and caspase-1 activities but authors could not find evidence of cannabinoid receptor involvement [232]. In some cases, activation of cell growth *via* a cannabinoid receptor-independent mechanism has also been described [231].

These studies, performed in different types of cells, demonstrate that some of the effects of cannabinoids are not mediated by CB_1 or CB_2 receptors. They could be mediated by non-specific interactions of cannabinoids with cells or by new unidentified receptors.

4. CONCLUDING REMARKS

During the last years our knowledge of the molecular mechanisms by which cannabinoids exert their actions in the organism has grown spectacularly. However, the exact biochemical pathways followed by cannabinoid receptors to regulate cellular functions are still unclear. As shown here, many intracellular pathways are activated by cannabinoids that include cell growth regulating cascades, cell death regulating systems, inflammatory mediators, metabolic pathways and gene transcription. Many cell responses to cannabinoids depend on agonist concentration, experimental conditions or specific tissue properties. Despite the extensive studies focused on signal transduction triggered by cannabinoid receptors, many questions remain to be solved. The near future undoubtedly will provide new insights into the complex actions by which cannabinoids regulate cell responses.

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