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Chemical name

SR141716A: *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide hydrochloride

Ceramide: a new second messenger of cannabinoid action

Manuel Guzmán, Ismael Galve-Roperh and Cristina Sánchez

Cannabinoids, the active components of *Cannabis sativa* (marijuana), and their endogenous counterparts exert their effects by binding to specific $G_{i/o}$-protein-coupled receptors that modulate adenylyl cyclase, ion channels and extracellular signal-regulated kinases. Recent research has shown that the CB_1 cannabinoid receptor is coupled to the generation of the lipid second messenger ceramide via two different pathways: sphingomyelin hydrolysis, and ceramide synthesis *de novo*. Ceramide in turn mediates cannabinoid-induced apoptosis, as shown by *in vitro* and *in vivo* studies. These findings provide a new perspective on how cannabinoids act, and raise exciting physiological and therapeutic questions.

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Marijuana, a well-known preparation from *Cannabis sativa* L., has been used for many centuries both medicinally and recreationally. However, the structure of its active components – the cannabinoids – was not determined until the early 1960s. Because of their high hydrophobicity, cannabinoids were thought to exert their actions by inserting into biomembranes. However, in the late 1980s, binding sites for cannabinoids were discovered in the brain[1]. Nowadays it is widely accepted that marijuana-derived cannabinoids act via specific receptors that are normally bound by a family of endogenous ligands: the endocannabinoids[2–5]. Two cannabinoid receptors have been characterized and cloned so far: CB_1 (Ref. 6) and CB_2 (Ref. 7). The central action and many of the peripheral effects of cannabinoids rely on activation of CB_1 receptors, which are particularly abundant in the nervous system but also present in various extra-neural sites. By contrast, CB_2 receptor expression is almost exclusively restricted to the immune system.

Extensive molecular and pharmacological studies have demonstrated that cannabinoid receptors are $G_{i/o}$-protein-coupled receptors that signal inhibition of adenylyl cyclase and activation of the extracellular signal-regulated kinase (ERK) cascade. Furthermore, the CB_1 receptor modulates ion channels, inducing, for example, inhibition of N- and P/Q-type voltage-sensitive Ca^{2+} channels and activation of G-protein-activated inwardly rectifying K^+ channels[8] (Fig. 1). In addition to these well-established G-protein-coupled events, recent observations have shown that CB_1 receptor activation triggers the generation of ceramide. This ubiquitous lipid second messenger plays an important role in the control of cell fate at different sites, including the CNS (Refs 9–11). Thus, exposure of neural cells to physical, chemical, bacterial or viral stimuli can increase the intracellular concentration of ceramide and therefore evoke changes in the decision between cell survival and cell death[9,10]. In addition, changes in ceramide metabolism exert important regulatory effects on neuronal growth and development[11]. Moreover, intracellular ceramide accumulation, which might in turn induce apoptotic cell death, has been shown to occur in neurodegenerative disorders such as Alzheimer's and Parkinson's disease, epilepsy and ischaemia (stroke)[9–11]. This new aspect of

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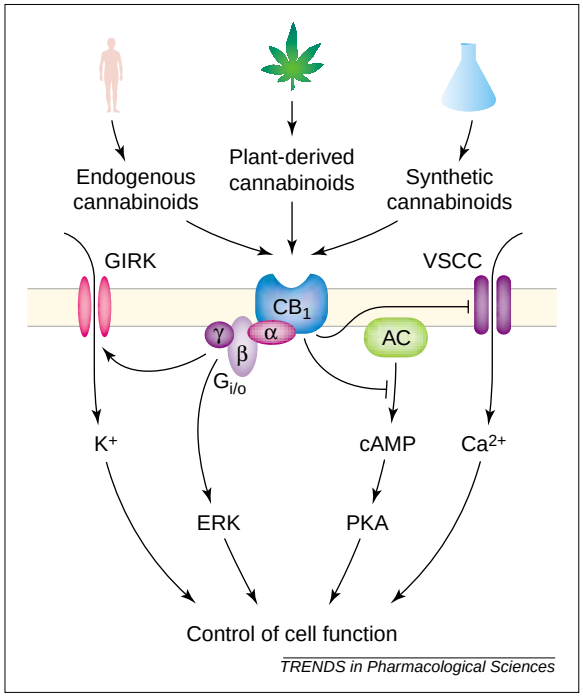

Fig. 1. G-protein-coupled signalling pathways activated by the cannabinoid CB_1 receptor. Endogenous, plant-derived and synthetic cannabinoids produce most of their effects by binding to the CB_1 receptor. This $G_{i/o}$-protein-coupled receptor signals inhibition of the adenylyl cyclase (AC)–protein-kinase-A (PKA) pathway and activation of the extracellular signal-regulated kinase (ERK) cascade. In addition, the CB_1 receptor modulates ion channels, inducing, for example, inhibition of N- and P/Q-type voltage-sensitive Ca^{2+} channels (VSCCs) and activation of G-protein-activated inwardly rectifying K^+ channels (GIRKs). All these events participate in the control of cell function by cannabinoids.

cannabinoid-mediated signal transduction and its potential physiological and therapeutic implications will be discussed.

Cannabinoid-induced acute ceramide generation

Mechanism

Ceramide generation that occurs within a time interval of minutes depends on the catalytic action of sphingomyelinases, which hydrolyse sphingomyelin to ceramide and phosphorylcholine[9]. CB_1 receptor activation induces sphingomyelin hydrolysis in both primary astrocytes[12,13] and C6 glioma cells[14,15], with a maximal effect after ~15 min. As expected, this stimulation is concomitant with an increase in ceramide concentrations (a maximum twofold increase after 15 min). The functional coupling of receptors to sphingomyelinases might involve different adaptor proteins, one of which is the factor associated with neutral sphingomyelinase activation (FAN). FAN binds to a cytoplasmic nine-amino-acid motif of the 55-kDa tumour necrosis factor (TNF) receptor, the neutral sphingomyelinase-activating domain, thereby coupling the receptor to sphingomyelin breakdown[16]. A role for FAN in CB_1-receptor-evoked sphingomyelin hydrolysis is supported by coimmunoprecipitation experiments that show FAN binding to the activated CB_1 receptor, and by evidence that cells expressing dominant-negative FAN are resistant to cannabinoid-induced sphingomyelin breakdown (C. Sánchez *et al.*, unpublished). Interestingly, both G-protein β-subunits and FAN are members of the WD-repeat protein family[16,17]. The WD repeat, which comprises a 44–60-amino-acid residue sequence that typically contains the WD dipeptide at the C-terminus, facilitates defined protein–protein interactions. In fact, many known WD-repeat proteins are regulatory or adaptor proteins involved in signal transduction[17].

changes in ceramide metabolism exert important regulatory effects on neuronal growth and development

The potential association of FAN with the CB_1 receptor is supported by the homology between a sequence in the 55-kDa TNF receptor FAN-binding domain (DSAHK) and a sequence in the cytoplasmic region of the CB_1 receptor (DCLHK). This homology is higher than that shared by the 55-kDa TNF receptor FAN-binding domain (EDSAH) and the sequence proposed to bind FAN in the immunoregulatory transmembrane protein CD40 (QETLH)[18]. Another G-protein-coupled receptor – the receptor for the chemokine growth-related gene product β – has been reported to evoke sphingomyelin hydrolysis through sphingomyelinase activation[19]. In short, cannabinoid-induced acute ceramide generation might rely on sphingomyelin breakdown via FAN (Fig. 2).

Function

The early peak of ceramide induced by cannabinoids has been linked to the regulation of metabolic functions (Fig. 2). Thus, cannabinoids stimulate the use of glucose (the main source of brain energy metabolism) and the production of ketone bodies (an alternative source of energy when glucose deprivation ensues) in primary astrocytes[20]. Both effects are prevented by CB_1 receptor antagonism and are preceded by a rapid and transient increase in ceramide levels. In turn, ceramide appears to mediate the stimulation of glucose consumption via the Raf1, mitogen-activated protein kinase ERK kinase (MEK), ERK cascade[12], and the stimulation of ketogenesis via the outer-mitochondrial-membrane carnitine palmitoyltransferase (the pace-setting enzyme for fatty acid oxidation)[13].

One of the most important functions of astrocytes is the regulation of brain energy metabolism by providing neurones with anaplerotic metabolites and substrates for generating energy. Therefore, it is tempting to speculate that, via ceramide, the endogenous cannabinoid system regulates the amount and type of nutrients supplied by astrocytes to neurones as a source of carbon for neuronal biosynthetic processes (e.g. myelination) and oxidative metabolism (e.g. synaptic activity). Further research is needed to clarify why CB_1 receptor activation in primary neurones does not stimulate sphingomyelin hydrolysis and glucose use (C. Sánchez *et al.*, unpublished).

Cannabinoid-induced sustained ceramide generation

Mechanism

Sphingomyelin hydrolysis is usually considered the normal mechanism of ceramide generation in ceramide

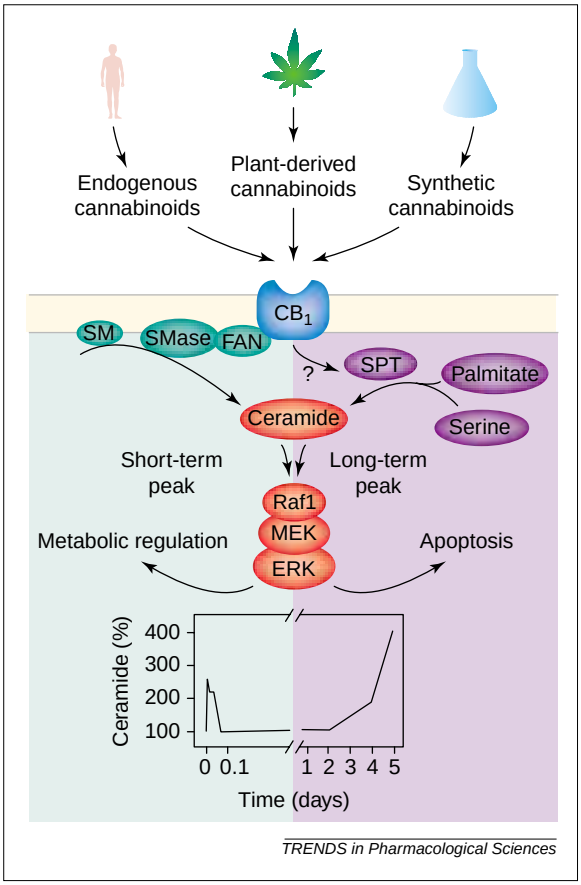

Fig. 2. The cannabinoid CB_1 receptor mediates ceramide accumulation. Activation of the CB_1 receptor can produce two peaks of ceramide. Short-term ceramide generation involves sphingomyelin (SM) hydrolysis via sphingomyelinase (SMase) activation possibly through the adaptor protein FAN (factor associated with neutral sphingomyelinase activation). Long-term ceramide generation might occur via serine palmitoyltransferase (SPT) induction and enhanced ceramide synthesis *de novo*. The two pools of ceramide elicit biological responses (e.g. metabolic regulation and apoptosis) through activation of the Raf1, mitogen-activated protein kinase extracellular signal-regulated kinase (ERK) kinase (MEK), ERK cascade. The plot shows the kinetics and magnitude of both peaks of ceramide in C6.9 glioma cells exposed for up to 5 days with 1 μM Δ^9-tetrahydrocannabinol, the main active component of marijuana[15].

signalling pathways. However, long-term ceramide accumulation through enhanced synthesis *de novo* or impaired clearance and/or metabolism have been gaining appreciation as alternative means of generating a signalling pool of ceramide[21]. In this context, cannabinoid receptor activation evokes sustained ceramide accumulation. In C6 glioma cells, Δ^9-tetrahydrocannabinol (THC), the main active component of marijuana, induces a second peak of ceramide starting at day 2–3 of treatment and reaching a maximal fourfold increase at day 5 (Ref. 15). Serine palmitoyltransferase, the pace-setting enzyme for ceramide synthesis *de novo*, might be involved in this effect, as indicated by measurements of enzyme activity and by the use of selective enzyme inhibitors (M. Guzmán *et al.*, unpublished). Other studies support the view that, like stress stimuli, G-protein-coupled receptors might induce apoptosis through enhanced ceramide synthesis *de novo*[22].

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In parallel to ceramide accumulation, ERK activation is detected in C6 glioma cells upon prolonged THC challenge[15]. Because ERK is one of the possible targets of ceramide action[9], the mechanism of ceramide-induced activation of ERK was investigated. Immunoprecipitation experiments showed that Raf1 was activated in cannabinoid-treated cells in which sustained ceramide production occurs, whereas in the same conditions, kinase suppressor of Ras, a different ceramide-activated protein kinase, was not activated[15] (Fig. 2).

Function

One of the most exciting aspects of current cannabinoid research is the possibility that these compounds participate in the control of the decision between cell survival and cell death[15,23]. The important role of ceramide in the induction of apoptosis prompted us to explore such a hypothesis. Experiments carried out using different C6 glioma cell subclones showed that sustained but not acute ceramide generation is responsible for cannabinoid-induced apoptosis[15]. Likewise, primary astrocytes, in which THC evokes acute but not sustained ceramide generation, are resistant to the apoptotic effect of the cannabinoid[12,14], but when ceramide synthesis *de novo* is induced selectively, they undergo apoptosis[24]. Moreover, primary neurones are resistant to cannabinoid-induced ceramide generation and apoptosis[14].

The essential role of the Raf1–MEK–ERK cascade as the target for *de novo* synthesized ceramide in the induction of apoptosis has been shown in both C6 glioma cells[15] and primary astrocytes[24]. The apoptotic effect of cannabinoids in glial cells might therefore rely on sustained ceramide accumulation and ERK activation (Fig. 2). It is worth noting that plant-derived and synthetic cannabinoids induce the regression of gliomas in laboratory animals, without significant side-effects[15]. There is a current renaissance in the study of the potential clinical use of cannabinoids[25]; one possibility is their use as therapeutic agents for the management of malignant brain tumours. In addition, because the endogenous cannabinoid system has been suggested to play a specific role in brain development[26], ceramide generated following CB_1 receptor activation might control neural cell fate during this process.

Concluding remarks

Recent investigations have shown that besides its well-known coupling to the modulation of adenylyl cyclase, ERK and ion channels through $G_{i/o}$ proteins, the CB_1 cannabinoid receptor is coupled to the generation of the lipid second messenger ceramide. Following CB_1 receptor activation two peaks of ceramide generation are observed that have different kinetics (minute- versus day-range), magnitude (two- versus fourfold), mechanistic origin (sphingomyelin hydrolysis versus ceramide synthesis *de novo*) and

function (metabolic regulation versus induction of apoptosis). These observations have changed our view of the mechanism of cannabinoid action, and contribute to the novel idea that G-protein-coupled receptors signal via mechanisms alternative to the classical heterotrimeric-G-protein paradigm[27].

In view of the emerging role of ceramide in the control of cell fate, together with the increasing array of cell functions modulated by cannabinoids, we anticipate that the cannabinoid–ceramide connection might yield exciting physiological implications and therapeutic possibilities.

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