Cannabinoid-Mediated Elevation of Intracellular Calcium: A Structure-Activity Relationship

Gautham K. Rao and Norbert E. Kaminski

Department of Pharmacology & Toxicology (GKR, NEK), Center for Integrative Toxicology (GKR, NEK), and National Food Safety & Toxicology Center (GKR, NEK), Michigan State University, East Lansing, Michigan, 48824-1317

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*Corresponding author: Norbert E. Kaminski, Department of Pharmacology & Toxicology and Center for Integrative Toxicology, 315 Food Safety Building, Michigan State University, East Lansing, Michigan 48824-1317. Tel: 517-353-3786; Fax: 517-432-3218; e-mail: kamins11@msu.edu

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The abbreviations used are: 2-AG, 2-arachidonoylethanolamide; AA, arachidonic acid; AEA, arachidonylethanolamide (anandamide); BCS, bovine calf serum; [Ca^{2+}], intracellular calcium concentration; CBN, cannabinol; CP55,940, (-)-cis-3-[2-Hydroxy-[3,5-3H]-4-(1,1-dimethylheptyl)phenyl]-trans-4-(3-hydroxypropyl)cyclohexanol; DMSO, dimethylsulfoxide; HPB-ALL, human peripheral blood acute lymphoid leukemia; HU-210, (6aR,10aR)-3-(1,1-dimethylbutyl)-6a,7,10,10a-tetrahydro-6,6-dimethyl-6H-dibenzo[b,d]pyran-9-methanol; JWH-133, (6aR,10aR)-3-(1,1-dimethylbutyl)-6a,7,10,10a-tetrahydro-6,6,9-trimethyl-6H-dibenzo[b,d]pyran; ROC channel, receptor-
operated cation channel; RPMI 1640, Roswell Park Memorial Institute 1640; SOC channel, store-operated calcium channel; SK&F 96365, 1-[β-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl]-1H-imidazole; SR141716A, N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-H-pyrazole-3 carboxyamidehydrochloride; SR144528, N-[(1S)-endo-1,3,3,-trimethyl bicyclo [2,2,1] heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide; TG, thapsigargin; ∆⁹-THC, ∆⁹-tetrahydrocannabinol; VH, vehicle; WIN 55,212-2, (R)-(+)-[2,3-Dihydro-5-methyl-3[(4-morpholinyl)methyl]pyrrolo[1,2,3-de]-1,4-benzoazinyl]-(1-naphthalenyl) methanone mesylate salt.

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Abstract

This laboratory has reported previously that Δ⁹-tetrahydrocannabinol (Δ⁹-THC) and cannabinol (CBN) robustly elevate intracellular calcium ([Ca^{2+}]_i) in resting human and murine T cells, whereas CP55,940, a high-affinity ligand for CB1 and CB2, does not. In light of our previous studies, the objective of the present investigation was to examine the ability of various cannabinoid compounds to elevate [Ca^{2+}]_i in the CB2 receptor-expressing HPB-ALL human T cell line, and the dependence of structural similarity to Δ⁹-THC therein. The present studies demonstrate that CBN and HU-210, both tricyclic and in that respect structurally similar to Δ⁹-THC, elevate [Ca^{2+}]_i. The [Ca^{2+}]_i elevation elicited by both CBN and HU-210 was attenuated upon removal of extracellular calcium and upon pretreatment with SK&F96365, an inhibitor of receptor-operated cation channels. Also, pretreatment with either CB1 or CB2 receptor antagonists attenuated the CBN- and HU-210-mediated [Ca^{2+}]_i elevation. Further investigation of the dependence of Δ⁹-THC, CBN and HU-210 on cannabinoid receptors using splenocytes from wildtype and CB1⁻/⁻/CB2⁻/⁻ mice showed that the [Ca^{2+}]_i elevation elicited by all three tricyclic cannabinoids was independent of CB1 and CB2. Moreover, both the CB1 and CB2 receptor antagonists attenuated that rise in [Ca^{2+}]_i elicited by the tricyclic cannabinoids in the wildtype and CB1⁻/⁻/CB2⁻/⁻ mouse splenocytes. Taken together, the present investigation demonstrates that classical tricyclic cannabinoids with structural similarity to Δ⁹-THC elicit a robust influx of calcium in T cells putatively through ROC channels in a manner sensitive to the cannabinoid receptor antagonists, but independent of the CB1 and CB2 receptors.
Introduction

The immunosuppressive properties of plant-derived cannabinoids have been widely investigated (Schatz et al., 1993; Condie et al., 1996; Klein et al., 2000). \( \Delta^9 \)-tetrahydrocannabinol (\( \Delta^9 \)-THC), the most extensively investigated cannabinoid, exhibits a broad range of immunomodulatory activity. The immunomodulatory effects of \( \Delta^9 \)-THC include direct effects on T cell function as evidenced by altered mitogen-induced cell proliferation, suppressed accessory cell function in T cell-dependent antibody responses, and altered production of several T cell-derived cytokines (Schatz et al., 1993; Condie et al., 1996; Newton et al., 1998; Klein et al., 2000). Despite extensive investigation, the specific mechanism responsible for altered T cell function by cannabinoids remains poorly understood. However, since immune cells express both cannabinoid receptors CB1 and CB2, the mechanism by which cannabinoids elicit their immunomodulatory effects is generally believed to be mediated by one or both cannabinoid receptors (Klein et al., 2003; Howlett et al., 2004).

Recent studies of the cannabinoid-mediated effects in various systems, including the immune system, have produced an increasing body of evidence suggesting the existence of novel receptors and/or non-receptor cellular targets through which cannabinoids influence biological responses. Most notably, biochemical and pharmacological studies in the murine brain and in endothelial cells have provided the strongest indications yet for the existence of uncharacterized cannabinoid receptors (Breivogel et al., 2001; Wiley and Martin, 2002; Offertaler et al., 2003; Mo et al., 2004). In addition, previous
investigations from this and other laboratories have demonstrated CB1 and CB2 receptor-independent effects of cannabinoids in immune cells (Faubert Kaplan et al., 2003; Sancho et al., 2003; Kraft et al., 2004). The modulation of interleukin-2 production by cannabinoids in activated T cells, in particular, has been shown to be insensitive to treatment with the cannabinoid receptor antagonists (Jan et al., 2002; Faubert Kaplan et al., 2003; Rockwell and Kaminski, 2004). By contrast, in unstimulated T cells, Δ⁹-THC and CBN induced a robust elevation of intracellular calcium ([Ca²⁺]ᵢ), which was sensitive to both cannabinoid receptor antagonists, SR141716A (SR1) and SR144528 (SR2) (Faubert Kaplan et al., 2003; Rao et al., 2004). However, CP55,940, a high-affinity CB1/CB2 non-selective agonist, could not elevate [Ca²⁺]ᵢ, which raised the possibility that antagonism of the cannabinoid-mediated [Ca²⁺]ᵢ rise by SR1 and SR2 may not imply CB1- or CB2-dependence (Rao et al., 2004).

In T cells, the elevation of [Ca²⁺]ᵢ is an important signaling event. [Ca²⁺]ᵢ is involved in positive and negative regulation of the expression of several genes. The elevation of [Ca²⁺]ᵢ occurs in two distinct phases upon the activation of the T cell antigen receptor. The first rapid phase is characterized by a modest release of stored intracellular calcium, followed by a slower, yet larger, second phase produced from extracellular calcium ([Ca²⁺]ₑ) influx (Lewis, 2001; Winslow et al., 2003). The influx of [Ca²⁺]ₑ, which accounts for the bulk of the [Ca²⁺]ᵢ elevation, is attributed to a highly specialized variety of store-operated calcium channel known as the calcium-release activated calcium channel. Once the [Ca²⁺]ᵢ levels are elevated, transcription factors such as NFAT are activated and can proceed to modulate gene expression (Feske et al., 2001; Lewis, 2001).
Conversely, if [Ca^{2+}]_i is elevated in T cells prior to cellular activation, T cells can enter a state of non-responsiveness known as T cell anergy (Harding et al., 1992; Nakayama et al., 1992; Schwartz, 1992; Nghiem et al., 1994; Faubert Kaplan et al., 2003).

The critical role for [Ca^{2+}]_i in T cells coupled with our prior observation that the cannabinoid-mediated [Ca^{2+}]_i elevation was putatively CB1- and CB2-dependent prompted the present characterization of [Ca^{2+}]_i elevation by various cannabinoids. The objective of the present investigation was to examine whether a structure-activity relationship exists for cannabinoid-induced [Ca^{2+}]_i elevation, and to elucidate the role of CB1 and CB2 receptors in this process. The importance of the investigation is especially critical toward determining whether the cannabinoid-induced effects are mediated through a specific or non-specific mechanism of action. Therefore, the present studies examined the mechanism of [Ca^{2+}]_i elevation by tricyclic cannabinoids in the CB2 receptor-expressing HPB-ALL human T cell line and splenocytes derived from wildtype and CB1^{-/-}/CB2^{-/-} mice. All experiments were performed in the absence of T cell activation in order to eliminate the confounding variable of T cell activation-dependent elevation in [Ca^{2+}]_i. The present studies demonstrate that classical tricyclic cannabinoids, compounds similar to Δ^9-THC, elevate [Ca^{2+}]_i. By contrast, cannabinoid ligands that are non-tricyclic did not elevate [Ca^{2+}]_i. Taken together, the present investigation demonstrates that “classical” tricyclic cannabinoids elevate [Ca^{2+}]_i robustly in T cells in a cannabinoid antagonist-sensitive, yet CB1 and CB2 receptor-independent manner through receptor-operated cation channels.
Methods

Compounds – Δ9-THC, CBN, CBD, CP55,940, 2-AG, AEA, SR144528 and SR141716A were provided by the National Institute on Drug Abuse. Arachidonic acid (AA) was purchased from Caymen Chemical Company (Ann Arbor, MI). WIN-55,212-2 was purchased from Sigma Chemical Company (St. Louis, MO). JWH-133 was from Tocris Cookson, Inc. (Ellisville, MO). HU-210 was a gift from Dr. Raphael Mechoulam (Hebrew University of Jerusalem, Israel).

Cell culture – The HPB-ALL human T cell line was generously provided by Dr. Jeffrey A. Ledbetter (Pacific Northwest Research Institute). HPB-ALL cells were cultured in RPMI 1640 medium (Gibco BRL, Grand Island, NY) supplemented with 100 units of penicillin/ml, 100 units of streptomycin/ml, 10% BCS (Hyclone, Logan, UT), 100 mM non-essential amino acids (Gibco BRL, Grand Island, NY) and 1 mM sodium pyruvate (Gibco BRL).

Animals - Pathogen-free female C57BL/6J mice, 6 weeks, were purchased from Charles River Breeding. On arrival, mice were randomized, transferred to plastic cages containing sawdust bedding (5 animals/cage) and quarantined for one week. Pathogen-free C57BL/6J CB1+/CB2+/ mice were generously provided by Dr. Andreas Zimmer (University of Bonn, Germany). C57BL/6J CB1+/CB2+/ mice were bred at the University Laboratory Animal Resources (Michigan State University). All mice were given food (Purina Certified Laboratory Chow) and water ad libitum. Mice were not
used for experimentation until their body weight was 17-20 g. Animal holding rooms were maintained at 21-24°C and 40-60% relative humidity with a 12 h light/dark cycle. All studies were performed in compliance with the Michigan State University AUCAUC Committee.

**Splenocyte preparation** – Splenocytes from C57BL/6J wildtype or CB1⁻/⁻/CB2⁻/⁻ mice were made into single cell suspensions and depleted of red blood cells using ACK lysing buffer (10 µM EDTA-Na₂, 10 mM KHCO₃, 150 mM NH₄Cl) for 5 min at RT. The cell suspension was washed repeatedly with RPMI 1640 medium. The collected leukocytes were then resuspended in Ca²⁺-KREB buffer for calcium determinations.

**Calcium determination** – Cells were washed twice in Ca²⁺-KREB buffer (129 mM NaCl, 5 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 1 mM CaCl₂, 5 mM NaHCO₃, 10 mM HEPES, 2.8 mM glucose, 0.2% BSA). For studies conducted in the absence of extracellular calcium, the Ca²⁺-KREB buffer was prepared as above without CaCl₂ and supplemented with 1 mM MgCl₂ and 20 µM EGTA. [Ca²⁺]ᵢ was determined by measuring the fluorescence of fura-2 dye, which is dually excited at 340 nm and 380 nm. Briefly, cells were incubated with cell-permeant fura-2 AM (1 µM, Molecular Research Products, Eugene, OR) for 30 min at 37°C in the dark. Cells were harvested, washed twice with Ca²⁺-KREB buffer to remove extracellular fura-2 dye, and adjusted to 5x10⁵ cells/ml (for HPB-ALL cells) or 5x10⁶ cells/ml (for splenocytes) in Ca²⁺-KREB buffer. Cells were placed in a 3 ml quartz cuvette with constant stirring. Calcium determinations were performed at room temperature with a Beckman Spex 1681 0.22m Spectrometer.
with dual excitation at 340 and 380 nm and emission at 510 nm (all slit widths were 1 mm). The dissociation constant for the fura-2-calcium complex was $1.45 \times 10^{-7}$ M. All compounds used in intracellular calcium determination were screened for autofluorescence using fura-2 sodium salt containing Ca$^{2+}$-KREB buffer. None of the compounds, with the exception of WIN 55,212-2, interfered with fura-2 measurements.
Results

Effect of CBN and HU-210 on $[Ca^{2+}]_i$ – Given our prior observation that $\Delta^9$-THC robustly elevates $[Ca^{2+}]_i$ in T cells (Rao et al., 2004), the primary focus of the present investigation was to determine whether a structure-activity relationship exists for cannabinoid-induced $[Ca^{2+}]_i$ elevation. Currently, both CBN and HU-210 (fig. 1A) elicited a robust and concentration-responsive elevation of $[Ca^{2+}]_i$ in the HPB-ALL cells, with a significant increase in $[Ca^{2+}]_i$ at concentrations greater than $10 \mu M$. However, in comparison to $\Delta^9$-THC, the magnitude of $[Ca^{2+}]_i$ elevation by both CBN (fig. 2A) and HU-210 (fig. 2B) at all concentrations (1-20 $\mu M$) was modest. At a concentration of 20 $\mu M$, the rise in $[Ca^{2+}]_i$ elicited by CBN ($185.5 \pm 50.4$ nM; n=4) and HU-210 ($200.3 \pm 13.9$ nM; n=3) were similar, and significantly smaller than the $[Ca^{2+}]_i$ elevation induced by 12.5 $\mu M$ $\Delta^9$-THC ($870.6 \pm 38.7$ nM; n=4). Interestingly, the $[Ca^{2+}]_i$ rise elicited by all three classical tricyclic cannabinoid compounds followed a significant time delay after injection of the cannabinoid into the cuvette. In addition, the time delay to onset of $[Ca^{2+}]_i$ elevation post injection varied depending on the cannabinoid and the concentration of cannabinoid applied to the cells. At a concentration of 20 $\mu M$, the time to onset of $[Ca^{2+}]_i$ elevation by CBN was $195.2 \pm 8.0$ s (n=5), whereas the time to onset of $[Ca^{2+}]_i$ elevation by HU-210 was at $342.1 \pm 7.6$ s (n=5). By contrast, a lower concentration of $\Delta^9$-THC (12.5 $\mu M$) took only $123.7 \pm 4.0$ s (n=8) to elicit a $[Ca^{2+}]_i$ elevation. It is intriguing to note that the time delay to onset of $[Ca^{2+}]_i$ elevation by CBN (20 $\mu M$) and $\Delta^9$-THC (12.5 $\mu M$) were similar, while HU-210 (20 $\mu M$) took significantly longer to induce an $[Ca^{2+}]_i$ rise. At 20 $\mu M$ the delay to onset to $[Ca^{2+}]_i$ rise for CBN and
HU-210 was shortest and resulted in a robust $[\text{Ca}^{2+}]_i$ elevation. Therefore, all successive calcium studies with CBN and HU-210 were performed at a concentration of 20 µM.

*Effect of $[\text{Ca}^{2+}]_e$ removal and SK&F96365 on CBN- and HU-210-induced $[\text{Ca}^{2+}]_i$ elevation* – Given the observation that both CBN and HU-210 robustly elevated $[\text{Ca}^{2+}]_i$, the mechanism by which the tricyclic cannabinoids induce $[\text{Ca}^{2+}]_i$ elevation was further investigated. HPB-ALL cells were treated with CBN or HU-210 either in the presence or absence of $[\text{Ca}^{2+}]_e$. Analogous to our prior observation for $\Delta^9$-THC, the absence of $[\text{Ca}^{2+}]_e$ strongly attenuated the elevation of $[\text{Ca}^{2+}]_i$ by both CBN (fig. 3A) and HU-210 (fig. 3B). Compared to the control experiment in the presence of $[\text{Ca}^{2+}]_e$, removal of $[\text{Ca}^{2+}]_e$ attenuated the magnitude of the $[\text{Ca}^{2+}]_i$ rise elicited by CBN and HU-210 by 85–88% (n=3). Moreover, in the absence of $[\text{Ca}^{2+}]_e$ both compounds elicited a very small and delayed increase in $[\text{Ca}^{2+}]_i$, which suggested strongly that the tricyclic cannabinoid-induced $[\text{Ca}^{2+}]_i$ elevation was independent of intracellular calcium stores. Therefore, the putative involvement of calcium store-independent receptor-operated cation (ROC) channels was investigated. It has previously been reported that SK&F96365 (SKF) is a potent inhibitor of ROC channels in the 10–50 µM concentration range (Merritt et al., 1990; Wu et al., 2005). Presently, calcium measurements were performed in cells treated with SKF (20–50 µM) for 300 s before treatment with cannabinoids. In these studies, SKF inhibited the rise in $[\text{Ca}^{2+}]_i$ elicited by both CBN (fig. 4A) and HU-210 (fig. 4B). Interestingly, SKF was differentially efficacious at inhibiting the CBN- and HU-210-induced rise in $[\text{Ca}^{2+}]_i$. At a concentration of 50 µM, SKF potently inhibited the CBN-induced elevation in $[\text{Ca}^{2+}]_i$ by 96.7 ± 1.2 % (n=3). At the same concentration, SKF was
less efficacious in inhibiting the HU-210-mediated rise in $[Ca^{2+}]_i$ as evidenced by a 47.5 ± 13.6 % attenuation (n=3). Another rather unusual effect of SKF pretreatment was that it reduced the time delay to onset of the HU-210-induced $[Ca^{2+}]_i$ elevation, but not the CBN-induced $[Ca^{2+}]_i$ elevation. Compared to vehicle control, the time delay to onset of $[Ca^{2+}]_i$ elevation for HU-210 in the presence of SKF (50 µM) was reduced by 165.3 ± 1.2 s (n=3).

**Effect of SR144528 and SR141716A on CBN- and HU-210-induced elevation in $[Ca^{2+}]_i$.**

Cannabinoids are widely reported to exert their biological activity through the two known cannabinoid receptors, CB1 and CB2. However, the $K_d$ values for cannabinoid agonists binding to both CB1 and CB2 are in the low to mid-nanomolar range (Pertwee, 1999b). In the present studies, an effect on $[Ca^{2+}]_i$ by tricyclic cannabinoids was not observed until micromolar concentrations were reached. To address the discrepancy as to whether CBN and HU-210 elicited $[Ca^{2+}]_i$ elevation occurs through the activation of the CB2 receptor, calcium measurements were performed in the presence of the CB2 receptor antagonist, SR2 (1-5 µM). Previous studies have shown that the $\Delta^9$-THC-induced $[Ca^{2+}]_i$ elevation in the HPB-ALL cells was sensitive not only to SR2, but also the CB1 receptor antagonist, SR1 (Rao et al., 2004). Therefore, parallel calcium measurements were also performed with SR1 (1-5 µM). HPB-ALL cells were pretreated with either SR2 or SR1 for 300 s prior to addition of cannabinoids. In the presence of either SR2 or SR1, a concentration-responsive attenuation of both the CBN- as well as the HU-210-mediated rise in $[Ca^{2+}]_i$ was observed (Table 1). Interestingly, whereas the elevation in $[Ca^{2+}]_i$ elicited by CBN was equally sensitive to both antagonists, the HU-210-induced $[Ca^{2+}]_i$
rise was more sensitive to SR1 than SR2. Compared to vehicle control, both SR2 (5 µM) and SR1 (5 µM) strongly attenuated the magnitude of [Ca2+]i rise elicited by CBN by 70.9 ± 3.4% (n=3) and 71.9 ± 2.3% (n=3), respectively. In contrast, the HU-210-induced [Ca2+]i rise was strongly attenuated by SR1 (5 µM; 97.6 ± 1.1% inhibition; n=3), but only modestly by SR2 (5 µM; 29.1 ± 6.9% inhibition; n=4).

Effect of CBD on [Ca2+]i in HPB-ALL cells – In addition to the tricyclic classical cannabinoids, the structure-activity relationship on [Ca2+]i elevation was also assessed in the HPB-ALL cells using a non-classical bicyclic cannabinoid, cannabidiol (CBD, fig. 1B). Apart from its structural dissimilarity to the tricyclic cannabinoids, CBD is considered not to act as an agonist at either the CB1 or CB2 receptors (Pertwee, 1999b). Treatment of HPB-ALL cells with CBD (1-20 µM), nonetheless, produced a small increase in [Ca2+]i (fig. 5A). However, in contrast to the [Ca2+]i elevation profiles of HU-210, CBN and Δ2-THC, the [Ca2+]i rise elicited by CBD was very modest, rapid, and not concentration-responsive (fig. 5A). In addition, the CBD-induced [Ca2+]i rise occurred in two distinct phases – a rapid first phase and a slower second phase. Biphasic [Ca2+]i elevation has previously been established in lymphocytes to be a characteristic of store-operated calcium entry (Parekh, 2003). To investigate the possibility that CBD was eliciting a release of stored intracellular calcium, cells were treated with CBD (10 µM) either in the presence or absence of [Ca2+]e. The results showed that although the first phase of the CBD-induced [Ca2+]i rise was maintained in the absence of [Ca2+]e, the second delayed phase was completely abolished (fig. 5B). The insensitivity of the first phase to [Ca2+]e indicates that CBD elicits a store-release, followed by [Ca2+]e influx.
Finally, the effect of SR2 on the CBD-induced \([\text{Ca}^{2+}]_i\) rise was assessed. Cells were pretreated with SR2 (5 \(\mu\)M) for 300 s prior to CBD (10 \(\mu\)M) addition. Unlike the \([\text{Ca}^{2+}]_i\) elevation elicited by CBN and HU-210, the CBD-mediated elevation of \([\text{Ca}^{2+}]_i\) was insensitive to SR2 (fig. 5C).

**Effect of other cannabinoids on \([\text{Ca}^{2+}]_i\)** – Apart from CBN, HU-210 and CBD, the synthetic cannabinoids, WIN55,212-2 and the CB2-selective agonist, JWH-133 (fig. 1B), were also tested for their ability to induce an influx in \([\text{Ca}^{2+}]_i\). The effect of WIN55,212-2 on \([\text{Ca}^{2+}]_i\) elevation could not be assessed due to its interference with fura-2 calcium measurements. On the other hand, JWH-133, a high affinity CB2-selective agonist, did not interfere with calcium measurements, but interestingly did not induce an elevation of \([\text{Ca}^{2+}]_i\) over a range of concentrations (0.1–20 \(\mu\)M) in the HPB-ALL cells (data not shown). Finally, the endocannabinoids (fig. 1C), 2-arachidonoylglycerol and arachidonylethanolamide (anandamide), along with the parent eicosinoid compound, arachidonic acid (AA), were also tested for their effect on \([\text{Ca}^{2+}]_i\) elevation. Neither the endocannabinoids (1-20 \(\mu\)M) nor AA (1-50 \(\mu\)M) elevated \([\text{Ca}^{2+}]_i\) in the HPB-ALL cells (data not shown).

**Effect of tricyclic cannabinoids on \([\text{Ca}^{2+}]_i\) in wildtype and CB1\(^{-/-}\)/CB2\(^{-/-}\) splenocytes** – To determine unequivocally whether the tricyclic cannabinoid compounds elevate \([\text{Ca}^{2+}]_i\) through the cannabinoid receptors, CB1 and CB2, \([\text{Ca}^{2+}]_i\) measurements were performed in splenocytes (SPLC) derived from C57BL/6J wildtype (WT) and CB1\(^{-/-}\)/CB2\(^{-/-}\) mice. All three tricyclic cannabinoids elicited a robust \([\text{Ca}^{2+}]_i\) rise in both WT and CB1\(^{-/-}\)/CB2\(^{-/-}\)
SPLC (fig. 6A, B, C). Similar to the above observation in the HPB-ALL cells of the cannabinoids evaluated, the $[\text{Ca}^{2+}]_i$ rise elicited by $\Delta^9$-THC (12.5 µM) was the most robust in magnitude (426.3 ± 49.4 nM in WT SPLC and 339.8 ± 94.2 nM in CB1$^{-/-}$/CB2$^{-/-}$ SPLC; n=5). By comparison, at a concentration of 20 µM, the $[\text{Ca}^{2+}]_i$ rise elicited by CBN (243.2 ± 39.5 nM in WT SPLC and 323.0 ± 64.2 nM in CB1$^{-/-}$/CB2$^{-/-}$ SPLC) and HU-210 (272.8 ± 27.7 nM in WT SPLC and 233.6 ± 40.1 nM in CB1$^{-/-}$/CB2$^{-/-}$ SPLC) was smaller. Moreover, there was no significant difference between the magnitude of $[\text{Ca}^{2+}]_i$ elevation elicited by any of the three cannabinoids in the WT compared with the CB1$^{-/-}$/CB2$^{-/-}$ SPLC (fig. 6A, B, C). Also consistent with the structure-activity relationship observed in the HPB-ALL cells, the time delay to onset of $[\text{Ca}^{2+}]_i$ rise varied with each cannabinoid. Somewhat surprisingly, the time delay to $[\text{Ca}^{2+}]_i$ elevation for 20 µM CBN (55.6 ± 3.2 s in WT SPLC; 50.1 ± 4.8 s in CB1$^{-/-}$/CB2$^{-/-}$ SPLC; n=5) was much shorter than for either 12.5 µM $\Delta^9$-THC (149.4 ± 7.6 s in WT SPLC; 132.5 ± 14.2 s in CB1$^{-/-}$/CB2$^{-/-}$ SPLC; n=5) or 20 µM HU-210 (140.8 ± 6.8 s in WT SPLC; 145.8 ± 6.1 s in CB1$^{-/-}$/CB2$^{-/-}$ SPLC; n=5). Once again, there was no significant difference in time delay between the WT and CB1$^{-/-}$/CB2$^{-/-}$ SPLC for any of the three compounds tested. Finally, consistent with the prior observation in B6C3F1 splenic T cells and HPB-ALL cells, the bicyclic cannabinoid, CP55,940, failed to elicit an elevation in $[\text{Ca}^{2+}]_i$ in either WT or CB1$^{-/-}$/CB2$^{-/-}$ SPLC (data not shown).

Effect of cannabinoid antagonists on $[\text{Ca}^{2+}]_i$ rise induced by tricyclic cannabinoids in wildtype and CB1$^{-/-}$/CB2$^{-/-}$ splenocytes – To pursue the premise that SR1 and SR2 antagonize the tricyclic cannabinoid-induced $[\text{Ca}^{2+}]_i$ elevation in a non-CB1 non-CB2
mediated manner, further [Ca\textsuperscript{2+}]\textsubscript{i} measurements were performed in CB1\textsuperscript{-}/CB2\textsuperscript{-} SPLC in the presence of SR1 or SR2. SPLC were treated with SR2, SR1 (1-5 \mu M) or VH for 300 s, followed by \Delta\textsuperscript{9}-THC (12.5 \mu M), CBN (20 \mu M) or HU-210 (20 \mu M). Remarkably, both antagonists attenuated the tricyclic cannabinoid-mediated increase in [Ca\textsuperscript{2+}]\textsubscript{i} in the WT as well as CB1\textsuperscript{-}/CB2\textsuperscript{-} SPLC (Table 2). Similar to the above observations in the HPB-ALL cells, SR2 and SR1 were differentially sensitive at antagonizing the [Ca\textsuperscript{2+}]\textsubscript{i} rise induced by different tricyclic cannabinoids in the WT and CB1\textsuperscript{-}/CB2\textsuperscript{-} SPLC. While the \Delta\textsuperscript{9}-THC-mediated [Ca\textsuperscript{2+}]\textsubscript{i} rise was sensitive to both SR2 and SR1 (Table 2; top panel), the HU-210-induced [Ca\textsuperscript{2+}]\textsubscript{i} rise could be antagonized significantly only by SR1, but not SR2, in both WT and CB1\textsuperscript{-}/CB2\textsuperscript{-} SPLC (Table 2; lower panel). Also, while SR1 was equally sensitive at antagonizing the HU-210-mediated [Ca\textsuperscript{2+}]\textsubscript{i} elevation in both models, the \Delta\textsuperscript{9}-THC-induced [Ca\textsuperscript{2+}]\textsubscript{i} rise was slightly more sensitive to SR1 than SR2 in the CB1\textsuperscript{-}/CB2\textsuperscript{-} SPLC. More interestingly, both SR2 and SR1 equally and significantly inhibited the CBN-induced [Ca\textsuperscript{2+}]\textsubscript{i} elevation only in the CB1\textsuperscript{-}/CB2\textsuperscript{-} SPLC, but not the WT SPLC (Table 2; middle panel).
Discussion

Previous work from this laboratory has established in various T cell preparations that plant-derived cannabinoids, ∆⁹-THC and CBN, elevated [Ca²⁺]ᵢ, whereas the high-affinity synthetic cannabinoid, CP55,940, did not (Faubert Kaplan et al., 2003; Rao et al., 2004). Furthermore, the elevation of [Ca²⁺]ᵢ by both ∆⁹-THC and CBN was antagonized by SR1 and SR2, and, therefore, deemed cannabinoid receptor-dependent (Faubert Kaplan et al., 2003; Rao et al., 2004). Using the HPB-ALL cells and SPLC from WT and CB1⁻/⁻/CB2⁻/⁻ mice, the present studies reexamined the premise that CB1 and CB2 receptors are involved in the cannabinoid-mediated elevation in [Ca²⁺]ᵢ in T cells. In addition, these studies also characterized the structure-activity relationship of various cannabinoids on the elevation of [Ca²⁺]ᵢ.

The current characterization provides several lines of evidence to suggest that CBN and HU-210 elevate [Ca²⁺]ᵢ in a manner similar to the structurally-related cannabinoid, ∆⁹-THC. First, both CBN and HU-210 elevate [Ca²⁺]ᵢ in a concentration-responsive manner, which is sensitive to SR1 and SR2. Second, removal of [Ca²⁺]ₑ and pretreatment with ROC channel blocker, SKF, resulted in attenuating the [Ca²⁺]ᵢ elevation induced by either CBN and HU-210, as has been shown recently with ∆⁹-THC (Rao and Kaminski, in press). Finally, all three tricyclic cannabinoids elicited a [Ca²⁺]ᵢ elevation that was not significantly different between the WT and CB1⁻/⁻/CB2⁻/⁻ SPLC.
Despite the apparent similarities in the profile of [Ca\textsuperscript{2+}]\textsubscript{i} elevation by Δ\textsuperscript{9}-THC, CBN and HU-210, many differences also exist. One factor differentiating the [Ca\textsuperscript{2+}]\textsubscript{i} responses of the three tricyclic cannabinoids is sensitivity to SR1 and SR2. While the CBN- and Δ\textsuperscript{9}-THC-induced [Ca\textsuperscript{2+}]\textsubscript{i} elevation was attenuated by both antagonists with similar sensitivities, the HU-210-mediated [Ca\textsuperscript{2+}]\textsubscript{i} elevation was more sensitive to SR1 than to SR2, even in the HPB-ALL cells, which do not express CB1 (Schatz et al., 1997; Rao et al., 2004). Second, the time delay to onset of [Ca\textsuperscript{2+}]\textsubscript{i} elevation varied for the different cannabinoids. The time delay to onset of [Ca\textsuperscript{2+}]\textsubscript{i} rise was dependent on the cell model, the compound, and the concentration applied to the cells. At present, the significance of the varying time delays to onset of [Ca\textsuperscript{2+}]\textsubscript{i} rise is unclear, but may represent a delay in signaling cascades. A final difference between the [Ca\textsuperscript{2+}]\textsubscript{i} responses of the three cannabinoids is their sensitivity to the ROC channel inhibitor, SKF. Currently, the CBN-induced [Ca\textsuperscript{2+}]\textsubscript{i} elevation was also shown to be strongly attenuated by SKF, as has been previously shown with Δ\textsuperscript{9}-THC (Rao and Kaminski, in press). However, the HU-210-mediated [Ca\textsuperscript{2+}]\textsubscript{i} was only weakly attenuated by SKF pretreatment.

Concerning the mechanism by which CBN and HU-210, elevate [Ca\textsuperscript{2+}]\textsubscript{i}, it is clear that the intracellular calcium stores and store-operated calcium channels are not involved. However, it is intriguing that both SR1 and SR2 attenuated the [Ca\textsuperscript{2+}]\textsubscript{i} elevation elicited by CBN and HU-210 in the CB2-expressing HPB-ALL cells. The ability of SR1 to attenuate [Ca\textsuperscript{2+}]\textsubscript{i} elevation in this model suggests that both SR1 and SR2 may antagonize an unknown cellular target, possibly a yet unidentified cannabinoid receptor subtype in T cells. Given the results of [Ca\textsuperscript{2+}]\textsubscript{i} measurements in the WT and CB1\textsuperscript{-/-}/CB2\textsuperscript{-/-} SPLC, it is
clear that the tricyclic cannabinoid-mediated elevation in \([Ca^{2+}]_i\) is independent of CB1 and CB2, but can be antagonized by both SR1 and SR2.

The present studies provide several broad insights to allude to the involvement of a yet unknown cannabinoid receptor in T cells. First, the concentrations of cannabinoids and cannabinoid antagonists required to elicit and antagonize the \([Ca^{2+}]_i\) elevation, respectively, exceed the binding affinities for either CB1 or CB2 by several orders of magnitude. For example, HU-210 has much higher affinity for the CB2 receptor, than CBN or \(\Delta^9\)-THC (Pertwee, 1999a; Berdyshev, 2000; Klein et al., 2003), but the efficacy of HU-210 to elicit a robust \([Ca^{2+}]_i\) elevation was about the same as CBN. On the other hand, \(\Delta^9\)-THC was more efficacious at eliciting a rise in \([Ca^{2+}]_i\) than either CBN or HU-210. To our knowledge, HU-210 has not been previously reported to be less efficacious than CBN or \(\Delta^9\)-THC at either the CB1 or CB2 receptors. In addition, JWH-133, a high-affinity CB2-selective agonist, did not induce a \([Ca^{2+}]_i\) elevation in the HPB-ALL cells even at a concentration of 20 \(\mu\)M. Finally, the lack of difference in the \([Ca^{2+}]_i\) rise elicited by tricyclic cannabinoids in WT and CB1\(^{-/-}\)/CB2\(^{-/-}\) SPLC provide unambiguous evidence to suggest that targets distinct from CB1 and CB2 exist in immune cells.

Given the current results that only \(\Delta^9\)-THC, CBN and HU-210 robustly elevate \([Ca^{2+}]_i\) in T cells, it is tempting to speculate the \([Ca^{2+}]_i\) elevation is a property unique to tricyclic cannabinoids possessing a pyran ring. It must be noted, however, that another tricyclic cannabinoid, JWH-133, which also possesses a pyran ring failed to elicit a rise in \([Ca^{2+}]_i\). Currently, it is postulated that only cannabinoids that possess a \(\Delta^9\)-THC-like three-ring
structure are able to robustly increase $[\text{Ca}^{2+}]_i$. It is also apparent from the present studies that certain non-tricyclic cannabinoids, such as CBD, may also elevate $[\text{Ca}^{2+}]_i$. Compared to its tricyclic counterparts, however, the CBD-induced $[\text{Ca}^{2+}]_i$ rise was not concentration-responsive, very modest in magnitude, rapid, biphasic, insensitive to SR2, and involved both calcium store-release and extracellular influx phases.

Based on the available data and the sensitivity to SR1 and SR2, previous publications from this laboratory had logically concluded that the cannabinoid-mediated $[\text{Ca}^{2+}]_i$ elevation was dependent on the CB1 and CB2 receptors (Faubert Kaplan et al., 2003; Rao et al., 2004). By contrast, the present studies using WT and CB1$^{-/-}$/CB2$^{-/-}$ SPLC clearly indicate that previous conclusions on the dependence of CB1 and CB2 for $[\text{Ca}^{2+}]_i$ elevation were incorrect based on the unanticipated off-target effects elicited by SR1 and SR2. The current studies argue that tricyclic cannabinoids induce a robust influx of $[\text{Ca}^{2+}]_e$, in a SR1- and SR2-sensitive, but CB1 and CB2 receptor-independent manner. Although the precise mechanism through which cannabinoids elevate $[\text{Ca}^{2+}]_i$ remains largely elusive, our studies effectively show that measurement of $[\text{Ca}^{2+}]_i$ is one functional response that may be utilized to characterize the putative non-CB1 non-CB2 cannabinoid receptor(s) in T cells. Another unique insight provided by the present studies is that different cannabinoids may act via diverging mechanisms of action to elevate $[\text{Ca}^{2+}]_i$ in T cells. Overall, these structure-activity relationship studies may provide the mechanistic foundation for the development of novel immunomodulatory cannabinoid therapeutics that lack psychotropic properties.
References


Footnotes

This work was supported by funds from National Institute of Drug Abuse Grants DA07908, DA020402 and DA016828.
Figure Legends

**Figure 1. Molecular structures of cannabinoid compounds.** (A) Structures of tricyclic classical cannabinoids: Δ⁹-THC, Δ⁹-tetrahydrocannabinol; HU-210, (-)-11-hydroxydimethylheptyl-Δ⁸-tetrahydrocannabinol; and CBN (cannabinol). (B) Structures of bicyclic cannabinoids and JWH-133: CP55,940, (-)-cis-3-[2-Hydroxy-[3,5-3H]-4-(1,1-dimethylheptyl)phenyl]-trans-4-(3-hydroxypropyl)cyclohexanol; CBD, cannabidiol; and JWH-133, (6aR,10aR)-3-(1,1-dimethylbutyl)-6a,7,10,10a-tetrahydro-6,6,9-trimethyl-6H-dibenzo[b,d]pyran. (C) Structures of arachidonic acid and endocannabinoids: AEA, arachidonylethanolamide (anandamide); and 2-AG (2-arachidonoylglycerol).

**Figure 2. Effect of CBN and HU-210 on \([Ca^{2+}]_i\), in the HPB-ALL cells.** A 3 ml aliquot of fura-2-AM loaded HPB-ALL cells (5x10⁵ cells/ml) was treated with various concentrations of CBN (A) or HU-210 (B) and/or vehicle (VH; 0.1% ethanol for CBN; 0.1% DMSO for HU-210) by injection into the cuvette and the increase in \([Ca^{2+}]_i\) was measured for 1600 s. For comparison, calcium elevation experiments were also performed under similar conditions with Δ⁹-THC (12.5 μM). \([Ca^{2+}]_i\) changes are presented as changes in the ratio of bound to free calcium (340 nm/380 nm). The calcium traces represent three to four independent experiments.

**Figure 3. Effect of CBN and HU-210 on \([Ca^{2+}]_i\), in the presence or absence of extracellular calcium (\([Ca^{2+}]_e\)).** A 3 ml aliquot of fura-2-AM loaded HPB-ALL cells (5x10⁵ cells/ml) were resuspended in either Ca²⁺-KREB (+[Ca²⁺]ₜ) or Ca²⁺-free KREB buffer (-[Ca²⁺]ₜ; see materials and methods) just prior to beginning calcium
measurements. At 300 s, CBN (20 µM; A) or HU-210 (20 µM; B) or vehicle (VH; 0.1% ethanol for CBN; 0.1% DMSO for HU-210) was injected into the cuvette and the increase in [Ca\textsuperscript{2+}]\textsubscript{i} was measured for 1600 s. The calcium traces are representative of three independent experiments.

**Figure 4.** CBN- and HU-210-mediated elevation in [Ca\textsuperscript{2+}]\textsubscript{i} is attenuated upon pretreatment with SK&F96365. A 3 ml aliquot of fura-2-AM loaded HPB-ALL cells (5x10\textsuperscript{5} cells/ml) was treated with SK&F96365 (SKF; 20-50 µM) or vehicle (VH; ddH\textsubscript{2}O) before beginning calcium measurements. At 300 s, CBN (20 µM; A) or HU-210 (20 µM; B) or vehicle (VH; 0.1% ethanol for CBN; 0.1% DMSO for HU-210) was injected into the cuvette and the increase in [Ca\textsuperscript{2+}]\textsubscript{i} was measured for 1600 s. The calcium traces represent three independent experiments.

**Figure 5.** Effect of CBD on [Ca\textsuperscript{2+}]\textsubscript{i} in the HPB-ALL cells. A 3 ml aliquot of fura-2-AM loaded HPB-ALL cells (5x10\textsuperscript{5} cells/ml) was treated either with various concentrations of CBD; in the presence or absence of [Ca\textsuperscript{2+}]\textsubscript{c}; or following a 300 s pretreatment with SR144528, and the increase in [Ca\textsuperscript{2+}]\textsubscript{i} was measured for 1600 s. (A) Cells were treated with varying concentrations of CBD (1-20 µM) or vehicle (VH; 0.1% ethanol) by injection into the cuvette. (B) Cells were resuspended in either Ca\textsuperscript{2+}-KREB (+[Ca\textsuperscript{2+}]\textsubscript{c}) or Ca\textsuperscript{2+}-free KREB buffer (-[Ca\textsuperscript{2+}]\textsubscript{c}) just prior to beginning calcium measurements. At 300 s, CBD (10 µM) or vehicle (VH; 0.1% ethanol) was injected into the cuvette. (C) Cells were treated with SR144528 (SR2; 5 µM) and/or vehicle (VH; 0.1% DMSO) before
beginning calcium measurements, followed by CBD (10 µM) at 300 s and the increase in
[Ca^{2+}]_i was measured for 1600 s.

**Figure 6.** Effect of ∆^{9-}THC, CBN and HU-210 on [Ca^{2+}]_i in WT and CB1^{−/−}/CB2^{−/−}
splenocytes. A 3 ml aliquot of fura-2-AM loaded in WT and CB1^{−/−}/CB2^{−/−} splenocytes
(5x10^6 cells/ml) was treated with ∆^{9-}THC (12.5 µM; A) CBN (20 µM; B) or HU-210 (20
µM; C) and/or vehicle (VH; 0.1% ethanol for ∆^{9-}THC and CBN; 0.1% DMSO for HU-
210) by injection into the cuvette and the increase in [Ca^{2+}]_i was measured for 1600 s.
[Ca^{2+}]_i changes are presented as changes in the ratio of bound to free calcium (340
nm/380 nm). The calcium traces represent at least three independent experiments.
Table 1. Effect of cannabinoid receptor antagonists on CBN- and HU-210-induced rise in 
\([\text{Ca}^{2+}]_i\) in HPB-ALL cells. A 3 ml aliquot of fura-2-AM loaded HPB-ALL cells (5x10^5 
cells/ml) was treated with SR141716A (SR1; 1-5 μM), SR144528 (SR2; 1-5 μM), and/or 
vehicle (VH; 0.1% DMSO) before beginning calcium measurements. At 300 s, CBN (20 
μM; left panel) or HU-210 (20 μM; right panel) or vehicle (VH; 0.1% ethanol for CBN; 
0.1% DMSO for HU-210) was injected into the cuvette and the increase in \([\text{Ca}^{2+}]_i\) was 
measured for 1600 s. \([\text{Ca}^{2+}]_i\) changes are presented as changes in the mean change in base 
to peak ratio ± SEM of bound to free calcium (340 nm/380 nm). The calcium data 
represent a mean of at least three independent experiments. *, P < 0.05 for paired 
comparison analysis (Dunnet’s test).
Table 1. Effect of cannabinoid receptor antagonists on CBN- and HU-210-induced rise in [Ca^{2+}]_i in HPB-ALL cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Δ Ratio (base to peak)</th>
<th>% of control</th>
<th>Treatment</th>
<th>Δ Ratio (base to peak)</th>
<th>% of control</th>
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<tbody>
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<td>VH</td>
<td>0.015 ± 0.004</td>
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<tr>
<td>CBN</td>
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<td>HU-210</td>
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*Significant difference compared to control.
Table 2. Effect of cannabinoid receptor antagonists on the Δ⁹-THC, CBN- and HU-210-induced rise in [Ca²⁺]ᵢ in WT and CB1⁻/⁻/CB2⁻/⁻ splenocytes. A 3 ml aliquot of fura-2-AM loaded WT and CB1⁻/⁻/CB2⁻/⁻ splenocytes (5x10⁶ cells/ml) was treated with SR141716A (SR1; 1-5 µM), SR144528 (SR2; 1-5 µM), and/or vehicle (VH; 0.1% DMSO) before beginning calcium measurements. At 300 s, Δ⁹-THC (12.5 µM; top panel), CBN (20 µM; middle panel) or HU-210 (20 µM; lower panel) or vehicle (VH; 0.1% ethanol for Δ⁹-THC and CBN; 0.1% DMSO for HU-210) was injected into the cuvette and the increase in [Ca²⁺]ᵢ was measured for 1600 s. [Ca²⁺]ᵢ changes are presented as changes in the mean change in base to peak ratio ± SEM of bound to free calcium (340 nm/380 nm). The calcium data represent a mean of at least three independent experiments. *, P < 0.05 for paired comparison analysis (Dunnet’s test).
Table 2. Effect of cannabinoid receptor antagonists on the ∆9-THC, CBN and HU-210-induced elevation in elevation of [Ca\textsuperscript{2+}]\textit{i} in wildtype and CB1\textsuperscript{-/}-CB2\textsuperscript{-/-} splenocytes

<table>
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<th>% of control</th>
<th>Treatment</th>
<th>∆ Ratio (base to peak)</th>
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<td></td>
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<td><strong>CB1\textsuperscript{-/}-CB2\textsuperscript{-/-}</strong></td>
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<tr>
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<tr>
<td>VH</td>
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Fig 1

A. Δ⁹-Tetrahydrocannabinol  
   Cannabinol  
   HU-210

B. CP55,940  
   Cannabidiol  
   JWH-133

C. N-Arachidonylethanolamide  
   2-Arachidonylglycerol  
   Arachidonic Acid
Figure 2B: Graph showing the ratio (340 nm/380 nm) over time (s) for different concentrations of Δ⁹-THC and VH.

Key:
- 12.5 μM Δ⁹-THC
- 20 μM
- 15 μM
- 10 μM
- 1 μM

Time (s): 0 - 1600
Ratio: 0 - 6.0
Figure 3B

Graph showing the ratio (340 nm/380 nm) over time for different conditions:
- HU-210 (+[Ca²⁺]₀)
- HU-210 (−[Ca²⁺]₀)
- VH (+[Ca²⁺]₀)
- VH (−[Ca²⁺]₀)

Time (s) ranges from 0 to 1500.