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Differential effects of cannabis extracts and pure plant cannabinoids on hippocampal neurones and glia

Duncan Ryan, Alison J. Drysdale, Roger G. Pertwee, Bettina Platt *

School of Medical Sciences, College of Life Sciences and Medicine, University of Aberdeen, Institute of Medical Sciences, Foresterhill, Aberdeen AB25 2ZD Scotland, UK

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Abstract

We have shown previously that the plant cannabinoid cannabidiol (CBD) elevates intracellular calcium levels in both cultured hippocampal neurones and glia. Here, we investigated whether the main psychotropic constituent of cannabis, Δ^9-tetrahydrocannabinol (THC) alone or in combination with other cannabis constituents can cause similar responses, and whether THC affects the responses induced by CBD. Our experiments were performed with 1 μM pure THC (pTHC), with 1 μM pure CBD (pCBD), with a high-THC, low CBD cannabis extract (eTHC), with a high-CBD, low THC cannabis extract (eCBD), with a mixture of eTHC and eCBD (THC:CBD = 1:1) or with corresponding 'mock extracts' that contained only pTHC and pCBD mixed in the same proportion as in eTHC, eCBD or the 1:1 mixture of eTHC and eCBD.

We detected significant differences in neurones both between the effects of pTHC and eTHC and between the effects of pCBD and eCBD. There were also differences between the Ca^{2+} responses evoked in both neurones and glia by eTHC and mock eTHC, but not between eCBD and mock eCBD. A particularly striking observation was the much increased response size and maximal responder rates induced by the mixture of eTHC and eCBD than by the corresponding 1:1 mixture of pTHC and pCBD.

Our data suggest that THC shares the ability of CBD to elevate Ca^{2+} levels in neurones and glia, that THC and CBD interact synergistically and that the cannabis extracts have other constituents yet to be identified that can significantly modulate the ability of THC and CBD to raise Ca^{2+} levels.

Keywords: Cannabinoids; THC; CBD; Calcium; Cell culture

The major psychoactive component found in the marijuana plant (*Cannabis sativa*), Δ^9-tetrahydrocannabinol (THC), is most renowned for its psychotropic effects, mediated via the body's endogenous cannabinoid (endocannabinoid) system. This comprises at least two cannabinoid receptors (CB_1 and CB_2) [21,11], with increasing evidence for the existence of others [23,20]. CB_1 receptors are located, in the main, in the central nervous system (CNS) and assumed to be responsible for THC's psychotropic actions (e.g. [26]). Distribution of the CB_1 receptor throughout the brain is not uniform, and most dense in areas such as the hippocampus, basal ganglia and cerebral cortex [10]. In contrast, CB_2 receptors were once thought to be found peripherally in cells of the immune system only [18]. However, expression of this receptor has now been shown in activated microglia [33,15,19], suggesting a possible involvement in CNS disease states. Moreover, recent evidence suggests neuronal CB_2 receptors in brainstem and cerebellum [29].

Caution regarding THC's medicinal potential lies in its psychoactive nature, since high concentrations may acutely have dramatic behavioural side effects (e.g. learning and memory disturbance [24]), and cause precipitation of mental disorders after long-term use [9]. Thus, some of the non-psychotropic cannabinoids have been suggested for therapeutic considerations, and when applied in combination may be able to counteract some of the unwanted side-effects of CB_1 acting compounds [14,19]. The non-psychoactive phytocannabinoid, cannabidiol (CBD), has a weak affinity for CB_1 receptors and does not act as an agonist at this receptor, but displays a number of therapeutically useful characteristics, e.g. anti-inflammatory effects [7,4], neuroprotective effects [28] and anticonvulsant effects [32] (for review, see [20,22]). However, the precise cellular mechanisms of CBD's actions, its interactions with THC, and the details of the involvement of the endocannabinoid system have yet to be fully demonstrated.

* Corresponding author. Tel. +44 1224 555741; fax: +44 1224 555719.
E-mail address: B.Platt@abdn.ac.uk (B. Platt).

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Recently, a number of clinical trials have been performed on cannabis-based compounds to test the so far mainly anecdotal evidence of their analgesic properties. For example, it was found that the cannabis-based drug Sativex® (GW Pharmaceuticals) was an effective analgesic in patients with central neuropathic pain [1]. This drug is a whole plant extract of *Cannabis sativa L.* containing both THC and CBD in approximately equal measure (see below). This and other cannabis extracts have undergone further clinical trials, for instance in patients with multiple sclerosis, with the assumption that they may offer additional benefits compared to the pure compounds [25,31,2]. Accordingly, in the present study, a comparison of a THC-rich (Tetranabinex®) and a CBD-rich (Nabidiolex®) extract with their pure counterparts was conducted on cultured hippocampal neurones. We focussed on the observation that pure CBD can modulate intracellular Ca^{2+} in hippocampal tissue [6], and conducted a comparative study for pure compounds, extracts and 1:1 mixtures of both. Primary hippocampal cultures were prepared from Lister-Hooded rat pups (1–3 days old), euthanised by cervical dislocation, in accordance with Home Office and institute regulations as described previously [6]. Briefly, the brain was quickly removed, the hippocampi dissected out and placed in ice-cold filtered HEPES buffered solution (HBS, composition in mM: NaCl 130; KCl 5.4; $CaCl_2$ 1.8; $MgCl_2$ 1; HEPES 10; glucose 25, compounds from Sigma, Pool, UK). Hippocampal tissue was treated with 1 mg/ml type X and XIV protease solution (Sigma, 40 min), washed and triturated. Following centrifugation and re-suspension in tissue culture medium (90% minimum essential medium (MEM) (Gibco, Paisley, UK) with 10% foetal bovine serum (Helena Biosciences, Sunderland, UK) and 2 mM L-glutamine (Sigma)), cells were plated in culture dishes (Gibco). Cultures were kept in a humidified incubator (37 °C; 5% CO_2) and allowed to mature for 2 days before replacement of MEM with neurobasal medium (Gibco, Paisley, UK), supplemented with 2% B27, 2 mM L-glutamine (Sigma), and 25 μM L-glutamate. Culture dishes were used for imaging at 5–10 days *in vitro*.

For calcium imaging experiments, cultures were washed with HBS and loaded with the cell-permeable fluorescent calcium indicator Fura-2-AM (10 uM, Molecular Probes, USA) for 1 h in the dark. The sodium channel blocker tetrodotoxin (TTX, 0.5 μM, Alomone, Jerusalem, Israel) was added to all perfusion media. Cultures were perfused with low Mg^{2+} HBS (composition in mM: NaCl 130; KCl 5.4; $CaCl_2$ 1.8; $MgCl_2$ 0.1; HEPES 10; glucose 25, compounds from Sigma, UK), at a rate of 1–2 ml/min, using a gravity perfusion system.

Ratiometric imaging was conducted using two different imaging systems fitted onto Olympus microscopes. The first system utilised a monochromator illumination system (Spectromaster 1, Perkin Elmer), controlled by the Oracal software (version 1.86, Life Sciences Resources Ltd.). The second system was controlled via the Improvision software package Openlab (version 4.03) using a DG-4 illumination system (Sutter Instruments, USA) and Hamamatsu Orca-ER CCD camera. Reproducibility of results was confirmed by testing all cannabinoids acutely on both systems.

During experimentation, a greyscale transmission image was initially captured. The ratio of the fluorescence excited at 380 and 340 nm was plotted over time after background fluorescence subtraction for all regions of interest (ROIs), with frame capture rates set at 5 s.

Pure CBD and THC (stored as 1mg/ml ethanolic stock solution) as well as cannabis extracts were a gift of GW Pharmaceuticals (UK). The 50 mg/ml ethanolic stock solution of the THC-rich extract (Tetranabinex®) contained 72.6% THC and 2.5% CBD, whilst the CBD-rich extract (Nabidiolex®) contained 64.6% CBD and 2.5% THC. The remaining percentage of both extracts contained minor cannabinoids (5–6%), terpenoids (6–7%), sterols (6%), triglycerides, alkanes, squalene, tocopherol, carotenoids and other minor, plant-derived components [27].

In each case, ethanol was evaporated off and the cannabinoids re-suspended in DMSO (at a cannabinoid concentration of 1 mM). The concentration of extracts applied was calculated to yield equimolar concentrations (1 μM) of pure THC (for eTHC) and pure CBD (for eCBD). Mock samples of extracts were also made, using only the ratio of THC to CBD. Thus, for mock-THC-rich extract, pure THC and CBD were added together in a ratio of 29.1:1 (i.e. 1 μM THC:0.034 μM CBD, as in Tetranabinex®) and for the mock CBD-rich extract, a ratio of CBD to THC of 25.9:1 (i.e. 1 μM CBD: 0.039 μM THC, as in Nabidiolex®). In addition to this, a 1:1 mixture of both pure THC and pure CBD was used to test if combining the two cannabinoids would yield additive effects. This contained 1 μM of both compounds (i.e. a 1:1 ratio). Similarly, a 1:1 ratio of the two genuine extract compounds was tested (again, containing ∼1 μM of both THC and CBD extracts).

The following abbreviations are used throughout this manuscript: pCBD = pure CBD, eCBD = CBD-rich extract, M-eCBD = mock CBD-rich extract, pTHC = pure THC, eTHC = THC-rich extract, M-eTHC = mock THC-rich extract, pure mix (1:1) = pCBD + pTHC (1 μM of each) & extract mix (1:1) = eCBD + eTHC (1 μM of each)).

N-Methyl-D-aspartate (NMDA, 50 μM with 100 μM glycine; stock: 10 mM in double-distilled water) was applied in every experiment to distinguish between neuronal and glial cells (for sample, see Fig. 1).

For data analysis, fluorescent units were converted into %ΔF/F, with F defined as an average of five baseline values before drug application. All experiments were performed a minimum of three times on different cultures. Data were exported to Excel and statistical analysis performed using Prism (version 4). As in previous studies, normality tests confirmed absence of normal distribution of data. Therefore, a Kruskal-Wallis test with a Dunn's post test (multiple drug groups vs. control) was used for multiple group comparisons. Percentage responders were calculated based on morphological criteria and NMDA responses, as previously described [6]. Significance was set at $P < 0.05$ = significant; $P < 0.01$ = highly significant; $P < 0.001$ = very highly significant.

As in our previous study [6], acute application of CBD, but also of all other cannabinoid compounds tested, evoked Ca^{2+} responses in both neurones (Fig. 1) and glia (Fig. 2).