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Δ^9 -tetrahydrocannabivarin impairs epithelial calcium transport through inhibition of TRPV5 and TRPV6

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ABSTRACT

Compounds extracted from the cannabis plant, including the psychoactive Δ^9 -tetrahydrocannabinol (THC) and related phytocannabinoids, evoke multiple diverse biological actions as ligands of the G protein-coupled cannabinoid receptors CB1 and CB2. In addition, there is increasing evidence that phytocannabinoids also have non-CB targets, including several ion channels of the transient receptor potential superfamily. We investigated the effects of six non-THC phytocannabinoids on the epithelial calcium channels TRPV5 and TRPV6, and found that one of them, Δ^9 -tetrahydrocannabivarin (THCV), exerted a strong and concentration-dependent inhibitory effect on mammalian TRPV5 and TRPV6 and on the single zebrafish orthologue drTRPV5/6. Moreover, THCV attenuated the drTRPV5/6-dependent ossification in zebrafish embryos *in vivo*. Oppositely, 11-hydroxy-THCV (THCV-OH), a product of THCV metabolism in mammals, stimulated drTRPV5/6-mediated Ca^{2+} uptake and ossification. These results identify the epithelial calcium channels TRPV5 and TRPV6 as novel targets of phytocannabinoids, and suggest that THCV-containing products may modulate TRPV5- and TRPV6-dependent epithelial calcium transport.

1. Introduction

Extracts from the plant *Cannabis sativa* contain a large number of different compounds derived from a diterpene structure, generally known as phytocannabinoids [1]. The best characterised phytocannabinoid is Δ^9 -tetrahydrocannabinol (THC), which is the main mediator of the psychoactive effects of marijuana, by acting as an agonist of the G protein-coupled cannabinoid receptor CB1 in the brain [2]. In addition to CB1 and the closely related CB2 [3], there is extensive evidence for other pharmacological targets of THC and related phytocannabinoids [4,5]. In particular, several members of the transient receptor potential (TRP) superfamily of cation channels have been identified as non-CB1/non-CB2 molecular targets of phytocannabinoids [6–9]. These include the thermosensory channels TRPM8 and TRPA1 as well as four heat-

activated members of the vanilloid subfamily, TRPV1, TRPV2, TRPV3 and TRPV4 [7,8,10,11]. Based on these findings and on the discovery that endocannabinoids such as anandamide or 2-arachidonoyl glycerol can activate TRPV1 and TRPV4 [12,13], TRP channels have been put forward as a class of ionotropic cannabinoid receptors that may potentially contribute to the diverse physiological effects of endo- and phytocannabinoids in the brain, gut, peripheral sensory system and skin [5,14,15].

In addition to the heat-activated TRPV1-TRPV4, the vanilloid subfamily contains two additional members, TRPV5 and TRPV6, which are functionally distinct in that they lack significant temperature sensitivity and exhibit an exquisite selectivity for calcium compared to monovalent cation channels [6,16–18]. The latter property lies at the basis of their physiological function as gate-keepers in calcium (re)absorbing

Abbreviations: TRPV, transient receptor potential vanilloid; THC, Δ^9 -tetrahydrocannabinol; THCV, Δ^9 -tetrahydrocannabivarin; CBDV, cannabidivarin; CBC, cannabichromene; CBD, cannabidiol; CBG, cannabigerol; CBGV, cannabigerovarin; THCV-OH, 11-hydroxy- Δ^9 -tetrahydrocannabivarin

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epithelia in kidney and gut [19]. Whether these calcium-selective TRPVs are modulated by phytocannabinoids has remained unknown. Here, we show that mammalian TRPV5 and TRPV6, as well as the single TRPV5/6 orthologue in zebrafish are potentially inhibited by Δ^9 -tetrahydrocannabinavarin (THCV). Moreover, we found that treatment of zebrafish larvae with THCV partially reproduces the bone formation deficit of the zebrafish *trpv5/6* loss-of-function mutant *matt-und-schlapp* [18]. Taken together, our results suggest that low micromolar concentrations of THCV can affect calcium homeostasis through inhibition of calcium-selective epithelial TRP channels.

2. Methods

2.1. Cells and cellular assays

HEK293 at passage numbers 15–30 were grown in DMEM containing 10% (v/v) fetal calf serum, 4 mM L-alanyl-L-glutamine, 100 U ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin at 37 °C in a humidity controlled incubator with 10% CO₂. Cells were transiently transfected with rabbit TRPV5, mouse TRPV6 or zebrafish TRPV5/6 constructs cloned in the bicistronic pCINeo/IRES/GFP vector using TransIT-293 transfection reagent. Experiments were performed between 16 and 24 h after transfection.

Currents were recorded in the whole-cell configuration of the patch-clamp technique using an EPC-9 amplifier and Patchmaster software (HEKA Elektronik, Lambrecht/Pfalz, Germany). Positively transfected cells were identified based on GFP fluorescence. Data were sampled at 5–20 kHz and digitally filtered off-line at 1–5 kHz. Currents were measured during 200-ms voltage-ramps from –150 to +100 mV applied at a rate of 0.5 Hz. Patch electrodes had resistances between 2 and 4 MΩ when filled with the intracellular solution, which contained (in mM): 150 NaCl, 5 EGTA, 5 MgCl₂, 10 Hepes, pH 7.2 with NaOH. Series resistances were between 3 and 10 MΩ and were compensated for 60–80 %, reducing voltage errors to less than 10 mV. The extracellular solution contained (in mM): 150 NaCl, 1 CaCl₂, 1 MgCl₂, 10 Hepes, pH 7.4 with NaOH. When indicated, CaCl₂ was omitted from this solution, to obtain a nominally calcium-free solution.

For intracellular Ca²⁺ measurements, transfected cells were incubated with 2 µM fura-2 acetoxymethyl ester for 30 min at 37 °C. Fluorescence was measured during alternating illumination at 340 and 380 nm using a Cell^M (Olympus, Aartselaar, Belgium) fluorescence microscopy system, and absolute calcium concentration was calculated from the ratio of the background-corrected fluorescence signals at these two wavelengths ($R = F_{340}/F_{380}$) as $[Ca^{2+}] = K_m \times (R - R_{min}) / (R_{max} - R)$, where K_m , R_{min} and R_{max} were estimated from *in vitro* calibration experiments with known calcium concentrations. The extracellular solution in these experiments contained (in mM): 150 NaCl, 6KCl, 1 MgCl₂, 10 Hepes, pH 7.4 with NaOH, supplemented with between 0 and 3.3 mM CaCl₂, as indicated.

2.2. Zebrafish ossification assay

Zebrafish (*Danio rerio*) embryos and larvae were obtained from a stable laboratory strain and raised at 28.5 °C on a 14 L : 10D photoperiod according to the Zebrafish Book [20]. Embryos were raised in E3 embryo medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂, 0.33 mM MgSO₄, 0.00001% Methylene Blue) until staged at 3 days post fertilization (dpf) following distinctive morphological markers [21], after which healthy larvae were placed in 24-well plates at a density of up to 10 larvae/ml/well and exposed to drug solutions. All the drugs were dissolved in ethanol and diluted with E3 media to the indicated concentrations maintaining a final vehicle concentration of 0.1%. During the exposure period the drugs were replaced daily and the larvae kept at 28.5 °C on a standard photoperiod. The number of ossified vertebral centers in 8dpf larvae was quantified by staining with alizarin red [22]. Control and treated larvae were placed on glass slide embedded in a 3%

(w/v) methylcellulose solution and then photographs were taken on a Leica DMI 6000B microscope.

2.3. Data and statistical analysis

Statistical analyses were performed using OriginPro 9 (OriginLab Corporation, Northampton, US). Group data are presented as mean ± SEM, or as individual data points (Fig. 3D). Group sizes are provided as n, representing the number of experimentally independent values. Normality of all datasets was evaluated using the Kolmogorov-Smirnov test, and when this test indicated that normality could not be rejected, statistical significance was determined using Student's two-tailed t-test, one-way or two-way ANOVA, as indicated. $P < 0.05$ was deemed to constitute the threshold for statistical significance. Tukey's *post hoc* test was used to compare groups when ANOVA yielded a main effect with $P < 0.05$.

2.4. Materials

Cannabinoids extracted and purified from *Cannabis sativa*, namely: THCV (Δ^9 -tetrahydrocannabinavarin), CBDV (cannabidivarin), CBC (cannabichromene), CBD (cannabidiol), CBG (cannabigerol), CBGV (cannabigerovarin) and THCV–OH (11-hydroxy- Δ^9 -tetrahydrocannabinavarin; produced via a synthetic route from the parent compound THCV) were provided by GW Pharma Ltd., Salisbury, UK. The cannabinoids were of > 95% purity. The chemicals used for preparing solutions for the cellular assays were supplied by Sigma-Aldrich (Overijse, Belgium), fura-2 acetoxymethyl ester by Gentaur Molecular Products (Brussels, Belgium), TransIT-293 transfection reagent by Mirus Bio (Madison, US), and cell culture reagents by Life Technologies (Merelbeke, Belgium).

3. Results

We used the whole-cell patch-clamp technique to evaluate the effect six non-THC cannabinoids, namely THCV, CBDV, CBC, CBD, CBG and CBGV, on mammalian TRPV5 and TRPV6 heterologously expressed in HEK293 cells. Under physiological ionic conditions, TRPV5 and TRPV6 are highly calcium-selective channels, but in the absence of calcium they mediate large monovalent currents [17,23,24]. The activity of both channels is tightly regulated by calcium-dependent negative feedback mechanism, whereby a rise in intracellular calcium inhibits channel activity. In view of these properties, we performed patch-clamp experiments using calcium-free intra- and extracellular solutions, resulting in stable, inwardly rectifying currents mediated by Na⁺ ions. After maximal development of the TRPV5- and TRPV6-mediated currents during cell dialysis with the EGTA-containing intracellular solution, the different cannabinoids were applied at a concentration of 10 µM, and their effect on the current amplitude at –80 mV was assessed after 3 min or steady-state inhibition. As shown in Fig. 1A,B, 10 µM THCV had a reversible inhibitory effect of on both TRPV5 and TRPV6. The inhibitory effect of THCV was concentration-dependent (Fig. 1C), with IC₅₀ values of 4.8 ± 0.6 µM and 9.4 ± 1.4 µM for TRPV5 and TRPV6, respectively. The effects of all other tested cannabinoids at a concentration of 10 µM did not reach statistical significance (Fig. 1D).

Zebrafish (*Danio rerio*) express one single orthologue of TRPV5/ TRPV6, and loss-of-function mutations in the *trpv5/6*-gene result in severe deficits in bone formation in the *matt-und-schlapp* mutant [18]. Therefore, zebrafish represent an instrumental *in vivo* model to evaluate the effect of modulating epithelial calcium channels on calcium homeostasis. As a first step, we performed whole-cell patch-clamp recordings in HEK293 cells expressing drTRPV5/6 to evaluate the effect of THCV. Notably, in comparison with its mammalian orthologues, drTRPV5/6-mediated currents in HEK293 are relatively stable in Ca²⁺-containing extracellular solution, thus allowing us to test the inhibitory

Fig. 1. Inhibition of monovalent TRPV5 and TRPV6 currents by THCV. (A) Representative time course (top) of inward currents at -80 mV in a HEK293 cell expressing rabbit TRPV5 following establishment of the whole-cell configuration (time 0) and upon application of $10 \mu\text{M}$ THCV, and current-voltage relations (bottom) at the indicated time points. (B) Same as (A), but in a cell expressing mouse TRPV6. (C) Concentration-response curve for the inhibition of TRPV5 and TRPV6 by THCV ($n = 5$ for each data point). (D) Summary of the inhibitory effect of different phytocannabinoids (all at $10 \mu\text{M}$) on TRPV5 and TRPV6 ($n = 5$ for each channel/cannabinoid combination). Horizontal full and dashed lines indicate mean $\pm$ SEM of vehicle control ($n = 5$). **, $P < 0.01$ versus vehicle control; one-Way ANOVA with Tukey's *post hoc* test.

effect of THCV in more physiological ionic conditions. In the presence of 1 mM Ca^{2+} , application of THCV at 1 and $10 \mu\text{M}$ induced a reversible inhibition of inward drTRPV5/6-mediated currents (Fig. 2A,C).

Like THC, the main metabolic pathway for THCV occurs via cytochrome P450-dependent oxidation, leading to the formation of 11-hydroxy tetrahydrocannabivarin (THCV-OH) [25,26]. We tested the effect of THCV-OH at a concentration of $10 \mu\text{M}$, and observed that, in contrast to THCV, it has a small but significant potentiating effect on drTRPV5/6-mediated currents (Fig. 2B,C).

Using Fura-2-based intracellular calcium imaging, we confirmed that THCV has a strong inhibitory effect on drTRPV5/6-mediated calcium uptake in HEK293 cells [18], whereas THCV-OH has a mild potentiating effect (Fig. 3A). Finally, we tested the effect of THCV and

THCV-OH on the development of the axial skeleton in zebrafish embryos. Embryos were treated with THCV (1 or $3 \mu\text{M}$) or THCV-OH ($3 \mu\text{M}$) from day 3 post fertilization (3 dpf), and ossification was assessed using alizarin red staining at 8 dpf (Fig. 3B) and quantified as the number of ossified vertebral centers [22]. In comparison to controls, embryos treated with THCV showed a significant reduction in the number of ossified vertebral centers, whereas THCV-OH resulted in an increased number of ossified vertebral centers (Fig. 3B).

4. Discussion

Our present results reveal that THCV acts as an antagonist, with low micromolar potency, of mammalian TRPV5 and TRPV6 as well as of

Fig. 2. Modulation of drTRPV5/6-mediated currents in Ca^{2+} -containing extracellular solution by THC and THC-OH. (A) Representative time course (left) of inward currents at -80 mV in a HEK293 cell expressing drTRPV5/6 following establishment of the whole-cell configuration (time 0) and upon application of $10 \mu\text{M}$ THC, and current-voltage relations (right) at the indicated time points. (B) Same as (A), but upon application of $10 \mu\text{M}$ THC-OH. (C) Summary of the effect of THC and THC-OH at -80 mV ($n = 5$ for each condition). *, ** and ***: $P < 0.05$, $P < 0.01$ and $P < 0.001$ versus vehicle control; unpaired two-tailed t-test.

zebrafish drTRPV5/6. Other tested cannabinoids (CBDV, CBC, CBD, CBG and CBGV) were ineffective at the tested dose of $10 \mu\text{M}$. The specific antagonistic effect of THC on mammalian TRPV5 and TRPV6 contrasts with the agonistic effect of THC and several other cannabinoids on the four other mammalian members of the vanilloid subfamily of TRP channels, TRPV1-TRPV4 [7,8]. However, inhibitory effects on the more distantly related cold- and menthol-activated channel TRPM8 have been reported for several cannabinoids, including THC inhibiting

the channel with an IC_{50} value of $\sim 0.9 \mu\text{M}$ [8].

Within the mammalian TRP channels, the epithelial calcium channels TRPV5 and TRPV6 stand out as the two most calcium-selective members, being almost exclusively permeable for calcium under physiological ionic conditions. They are both mainly expressed in the apical membrane of calcium-transporting epithelia, where they are postulated to act as gatekeepers for transcellular calcium transport [19]. In addition, TRPV6 expression is upregulated in prostate cancer and various

other malignancies, and it has been suggested that inhibition of TRPV6 may be the target of novel anticancer treatments [27]. Genetic ablation of either channel in mice leads to specific deficits in epithelial calcium handling. For instance, TRPV5 is highly expressed in Ca^{2+} -reabsorbing epithelia in the kidney, and, concomitantly, TRPV5-deficient mice exhibit renal calcium wasting, as well as mild disturbances in bone structures. TRPV6-deficient mice exhibit male infertility attributed to an insufficient calcium transport by epididymal epithelium, as well as reduced intestinal Ca^{2+} absorption when placed on a low- Ca^{2+} diet, and impaired maternal-fetal calcium transport. Overall, however, under normal breeding conditions both the TRPV5- and the TRPV6-deficient mice develop with no obvious anatomical abnormalities, indicative of the existence of partial functional redundancy and/or compensatory mechanisms regulating global Ca^{2+} homeostasis. It is likely that

combined elimination of TRPV5 and TRPV6 will lead to a much more severe disruption of Ca^{2+} homeostasis in mammals, but technical difficulties in creating combined TRPV5/TRPV6 double knockout mice have precluded a direct test of this hypothesis [27]. In contrast, genetic ablation in zebrafish of the single orthologue, drTRPV5/6, leads the lethal *matt-und-schlapp* phenotype [18]. This mutant is characterized by a severe reduction in organismal Ca^{2+} content and a full lack of ossification of the axial skeleton, which can be partially rescued by increasing the calcium content of the growth medium [18]. These findings indicated that drTRPV5/6 is essential for Ca^{2+} uptake, a process that mainly takes place in the gills and that is vital for zebrafish calcium homeostasis.

There is a growing interest in the use of phytocannabinoids for medical use. For instance, preclinical studies have provided evidence

that THCv has beneficial effects on glycemic control in models of type 2 diabetes, reduces pentylenetetrazole-induced seizures, and causes pain relief in inflammatory models [28–30]. These effects have generally been attributed to modulation of CB1 and/or CB2 receptors: *in vitro* displacement assays indicate that THCv binds to both CB receptors with affinities in two digits nanomolar range, and behaves as a neutral CB1 antagonist and, depending on the *in vitro* and *in vivo* assays used, a CB2 agonist or antagonist [5]. Our present results suggest that THCv, in addition to its effects on CB1/CB2, may also influence the various physiological processes in humans involving TRPV5/TRPV6-dependent Ca^{2+} transport. Using zebrafish embryos as a vertebrate *in vivo* model, we found that including 1–3 μM THCv in the medium caused a significant reduction in axial ossification, in line with its inhibitory effect on drTRPV5/6 currents and cellular calcium uptake, mimicking the *matt-und-schlapp* phenotype. In further support of the concept that drTRPV5/6 mediates the effect of THCv in zebrafish ossification, we observed that THCv–OH, a major metabolite of THCv in mammals, caused a significant potentiation of drTRPV5/6-mediated cellular Ca^{2+} uptake and enhanced ossification in the zebrafish embryo. Nevertheless, at this point we cannot fully exclude that the effects of THCv or THCv–OH on zebrafish ossification are dependent on other potential targets of THCv, such as the zebrafish orthologues of CB1 and CB2 [31,32].

Plasma concentrations of THCv in recreational cannabis users are typically in the low nanomolar range [33,34], which, based on our present results, would be too low to cause any significant effect on TRPV5 or TRPV6 activity. Likewise, the doses of THCv that have been used to ameliorate glycemic parameters or reduce inflammatory pain *in vivo* are unlikely to result in supra-micromolar THCv plasma concentrations [28,30,35]. Nevertheless, micromolar concentrations have been detected in plasma and brain of mice and rats treated i.p. or p.o. with 30 mg/kg of THCv [36], and the full pharmacokinetics of THCv upon repeated dosing are not known. Likewise, there is no quantitative information available on the levels of THCv–OH that occur in humans following THCv administration. Therefore, future work in mammalian models may be warranted to further explore potential effects of the use of THCv-based medicinal products on epithelial calcium transport and other TRPV5- or TRPV6-related processes in humans.

Conflict of interest

None.

Author contributions

AJ and TV performed and analysed patch-clamp and calcium imaging experiments, CS and AM performed and analysed zebrafish experiments, JVO provided essential experimental tools, TV and VDM designed experiments, TV coordinated the project and wrote the manuscript. All authors commented on the manuscript.

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