Inhibition of human recombinant T-type calcium channels by phytocannabinoids in vitro

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Background and Purpose: T-type Ca channels (I_{Ca}) regulate neuronal excitability and contribute to neurotransmitter release. The phytocannabinoids Δ^9-tetrahydrocannabinol and cannabidiol effectively modulate T-type I_{Ca}, but effects of other biologically active phytocannabinoids on these channels are unknown. We thus investigated the modulation of T-type I_{Ca} by low abundance phytocannabinoids.

Experimental Approach: A fluorometric (fluorescence imaging plate reader [FLIPR]) assay was used to investigate modulation of human T-type I_{Ca} (Ca_3.1, 3.2 and 3.3) stably expressed in FlpIn-TREx HEK293 cells. The biophysical effects of some compounds were examined using whole-cell patch clamp recordings.

Key Results: In the FLIPR assay, all 11 phytocannabinoids tested modulated T-type I_{Ca}, with most inhibiting Ca_3.1 and Ca_3.2 more effectively than Ca_3.3. Cannabigerolic acid was the most potent inhibitor of Ca_3.1 (pIC_{50} 6.1 ± 0.6) and Ca_3.2 (pIC_{50} 6.4 ± 0.4); in all cases, phytocannabinoid acids were more potent than their corresponding neutral forms. In patch clamp recordings, cannabigerolic acid inhibited Ca_3.1 and 3.2 with similar potency to the FLIPR assay; the inhibition was associated with significant hyperpolarizing shift in activation and steady-state inactivation of these channels. In contrast, cannabidiol, cannabidivarin, and cannabigerol only affected channel inactivation.

Conclusion and Implications: Modulation of T-type calcium channels is a common property of phytocannabinoids, which all increase steady-state inactivation at physiological membrane potentials, with some also affecting channel activation. Thus, T-type I_{Ca} may be a common site of action for phytocannabinoids, and the diverse actions of phytocannabinoids on channel gating may provide insight into structural requirement for selective T-type I_{Ca} modulators.

KEYWORDS
- cannabidivarin, cannabigerolic acid, electrophysiology, epilepsy, FLIPR, pain, phytocannabinoids, T-type calcium channels, Δ^9-tetrahydrocannabinol

Abbreviations: CBC, cannabichromene; CBDA, cannabidiolic acid; CBDV, cannabidivarin; CBDVA, cannabidivarinic acid; CBG, cannabigerol; CBGA, cannabigerolic acid; CBN, cannabinol; FLIPR, fluorescence imaging plate reader; THCA, Δ^9-tetrahydrocannabinolic acid; THCV, Δ^9-tetrahydrocannabivarin; TRPV, transient receptor potential receptor.

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Phytocannabinoids are naturally occurring compounds from Cannabis sativa (Gertsch et al., 2010) now being explored as pharmaceutical agents with potential applications in the treatment of pain, epilepsy, and inflammatory disorders of the gut among other indications (Gaston & Friedman, 2017; Hill et al., 2012; Izzo et al., 2009). Δ²-Tetrahydrocannabinol (THC), a low efficacy agonist of cannabinoid CB₁ and CB₂ receptors and cannabidiol (CBD), which inhibits signalling at CB₁ and CB₂ receptors (Pertwee, 2008), are the most abundant phytocannabinoids in Cannabis. CBD also ameliorates some perceived negative effects of THC in vivo (Russo & Guy, 2006).

Medicines containing THC and/or CBD have been approved for the treatment of nausea, spasticity associated with multiple sclerosis, chronic pain, and epilepsy in a number of countries (Sholler et al., 2020). In regions with legal medicinal Cannabis programmes, available evidence suggests patients consume Cannabis preparations predominantly to alleviate pain, spasticity, nausea, and other neurological conditions such as posttraumatic stress disorder and epilepsy (Henderson et al., 2021; Oregon Health Authority, OMMP Statistics, 2022). It seems likely that THC activity at CB₁ receptors contributes significantly to the reported therapeutic efficacy of Cannabis in people, but this has not been directly examined, and agonist actions at CB₂ receptors cannot explain the effects of cannabidiol.

More than 100 phytocannabinoids in addition to THC and CBD have been identified in Cannabis, of these only cannabiol (CBN) and nabichromene (CBC) have well-recognized agonist actions at CB₁/₂ receptors (Morales et al., 2017; Udoh et al., 2019). The therapeutic effects of less abundant phytocannabinoids have only begun to be explored; however, it is widely believed they might contribute to the potential medicinal activity of whole Cannabis preparations, and many biological activities for these compounds have been identified (Izzo et al., 2009; Morales et al., 2017). The facile synthesis of phytocannabinoids in yeast means that any of these compounds with useful pharmacological properties can be made at scale (Luo et al., 2019).

Phytocannabinoids also modulate ion channels. Transient receptor potential channels including TRPV1, TRPV2, and TRPA1 are modulated by phytocannabinoids including THC, Δ²-tetrahydrocannabinolic acid (THCA), CBD, and cannabinerolic acid (CBGA) (De Petrocellis et al., 2011; Jordt et al., 2004; Qin et al., 2008). Ligand-gated ion channels including 5-HT₃ receptors (Xiong et al., 2012), glycine receptors (Ahrens et al., 2009), and GABAₐ receptors are allosterically modulated by phytocannabinoids such as THC, CBD (Bakas et al., 2017), cannabidivarin (CBDV) (Morano et al., 2016), and Δ²-tetrahydrocannabinol (THCV) (Ma et al., 2008). CBD and cannabinerol (CBG) inhibit voltage gated Na⁺ channels (Ghovanloo et al., 2018; Hill et al., 2014) and CBD inhibits N-type (Ca₂,2) calcium channels (Mendis et al., 2019). Low-voltage activated T-type calcium channels (T-type I₄Ca) are modulated by THC and CBD as well as a variety of endocannabinoids and related molecules including anandamide, N-arachidonoyl dopamine, N-arachidonoyl 5-HT, and other N-acylethanolamines (Barbara et al., 2009; Chemin et al., 2001; Gilmore et al., 2012; Ross et al., 2009).

**What is already known**
- Cannabinoid receptor inactive phytocannabinoids are active in preclinical models of pain, inflammation and epilepsy.
- The phytocannabinoids THC and CBD both modulate T-type calcium channels, but do so differently.

**What does this study add**
- All phytocannabinoids we tested modulate T-type calcium channels, with phytocannabinoid acids most potent.
- Phytocannabinoids all enhance inactivation of the channels, some simultaneously promote channel activation.

**What is the clinical significance**
- Phytocannabinoids lacking psychoactivity effectively target ion channels important in pain, epilepsy, and gut disorders.
fluorescence assay which measures changes in intracellular Ca\(^{2+}\). We also used patch clamp electrophysiology to explore the effects of some phytocannabinoids on channel gating. All the phytocannabinoids tested modulated T-type \(I_{\text{Ca}}\) to some degree, and we also provide further evidence for distinct effects on channel gating for some phytocannabinoids.

2 | METHODS

2.1 | Transfection and cell culture

HEK 293 T-Rex cells (ThermoFisher, Melbourne, Australia) were stably transfected with plasmids encoding human Ca\(_V3\) channels in PDNA5 with pOG44 using Fugene HD as per Promega protocol. Cells were cultivated in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS and 1% penicillin–streptomycin. Cells were selected using 150 μg ml\(^{-1}\) Blasticidin (InvitroGen, Melbourne, Australia) and 100 μg ml\(^{-1}\) Hygromycin.

2.2 | Intracellular calcium measurements

Fluorescence imaging plate reader (FLIPR) assays were used for the detection of compounds acting on T-type calcium channels (Belardetti et al., 2009; Xie et al., 2007). Under our experimental conditions, the HEK293 cells we used did not require expression of channels other than the Ca\(_V3\) proteins to see robust fluorescent signals resulting from channel activation. HEK293 cells expressing Ca\(_V3\) were plated on poly-D-lysine pre-coated 96-well plates in L-15 media supplemented with 1% FBS, 1% penicillin–streptomycin, 15-mM glucose and incubated overnight at 37°C in humidified incubator. Channel expression was induced with 2 mg ml\(^{-1}\) tetracycline (Sigma-Aldrich, Castle Hill, Australia) at the time of plating. On the day of the assay, cells were loaded with Calcium 5 dye (FLIPR Ca Assay Kit, Molecular Devices, San Jose, CA) diluted with HBS buffer containing (in mM): 145 NaCl, 0.407 MgSO\(_4\), 1.26 CaCl\(_2\), 0.493 MgCl\(_2\), 22 HEPES, 5.5 glucose, 0.441 KH\(_2\)PO\(_4\), 4.17 NaHCO\(_3\), and 0.338 Na\(_2\)HPO\(_4\) (pH to 7.4 with NaOH, osmolarity = 330 mosm). The final concentration of DMSO was 0.1% and had no effect on T-type \(I_{\text{Ca}}\) function (Table 2). Recordings were sampled at 10 kHz and filtered at 1 kHz. Any cells that had a leak current that exceeded 50 pA were discarded. All currents were leak subtracted using P/N4 software package (Molecular Devices). Internal solution contained (in mM): 114 CsCl, and 5 BaCl\(_2\) (pH to 7.4 with CsOH, osmolarity = 330 mosm). The final concentration of DMSO was 0.1% and had no effect on T-type \(I_{\text{Ca}}\) function (Table 2). Recordings were sampled at 10 kHz and filtered at 1 kHz. Any cells that had a leak current that exceeded 50 pA were discarded. All currents were leak subtracted using P/N4 protocol. For measuring tonic inhibition of T-type \(I_{\text{Ca}}\), the membrane potential was stepped from −100 to −30 mV for at least 2 min, followed by washing out of all drugs with superfusion of control vehicle (external buffer) for approximately 3 min. Before determining the effects of drugs on activation and inactivation of Ca\(_V3\) channels, external control solution was applied for 5 min to minimize time-dependent changes in channel properties. Parallel vehicle controls were run for all experiments: vehicle (0.1% DMSO) no effect on Ca\(_V3\) channel kinetics (Table 2). In experiments measuring voltage dependence of activation, cells were held at −100 mV and depolarized from −75 to 50 mV, in 5-mV increments. For steady-state inactivation experiments, the membrane potential was depolarized by 3.6 s conditioning prepulses ranging from −110 to −20 mV and then stepped from −100 to −30 mV, for 20 ms. All experiments were repeated at least six times on separate cells.

2.3 | Electrophysiological recordings

Electrophysiological recordings were done at room temperature using Axopatch 200B amplifier (Molecular Devices Sunnyvale, CA, USA). Voltage commands and membrane currents were digitized and measured using a Digidata 1320 interfaced with Clampex 9.2 and pClamp software package (Molecular Devices). Internal solution contained (in mM): 126.5 CsMeSO\(_4\), 11 EGTA, and 10 HEPES, adjusted to pH 7.4 with CsOH. Just prior to experiments, 0.6-mM GTP and 2-mM ATP were added to the internal solution before use. The external solution was composed of (in mM): 1 MgCl\(_2\), 10 HEPES, 10 Glucose, 114 CsCl, and 5 BaCl\(_2\) (pH to 7.4 with CsOH, osmolarity = 330 mosm). The final concentration of DMSO was 0.1% and had no effect on T-type \(I_{\text{Ca}}\) function (Table 2). Recordings were sampled at 10 kHz and filtered at 1 kHz. Any cells that had a leak current that exceeded 50 pA were discarded. All currents were leak subtracted using P/N4 protocol. For measuring tonic inhibition of T-type \(I_{\text{Ca}}\), the membrane potential was stepped from −100 to −30 mV for at least 2 min, followed by washing out of all drugs with superfusion of control vehicle (external buffer) for approximately 3 min. Before determining the effects of drugs on activation and inactivation of Ca\(_V3\) channels, external control solution was applied for 5 min to minimize time-dependent changes in channel properties. Parallel vehicle controls were run for all experiments: vehicle (0.1% DMSO) no effect on Ca\(_V3\) channel kinetics (Table 2). In experiments measuring voltage dependence of activation, cells were held at −100 mV and depolarized from −75 to 50 mV, in 5-mV increments. For steady-state inactivation experiments, the membrane potential was depolarized by 3.6 s conditioning prepulses ranging from −110 to −20 mV and then stepped from −100 to −30 mV, for 20 ms. All experiments were repeated at least six times on separate cells.

2.4 | Data analysis

Changes in Ca fluorescence signals produced by drug alone were expressed as the percentage change in fluorescence from the baseline. To compare changes in channel activation produced by addition of 10 mM Ca\(^{2+}\), area under curve (AUC) analysis was used to compare the total change in fluorescence produced by activation of the Ca\(_V3\) channels in the absence and presence of drug. All data were analysed after subtraction of the changes produced by solvent (DMSO, 0.1%) alone. Data were analysed with PRISM (Graph Pad Software 8.02, San Diego USA) and values represent the mean ± SEM of at least six independent experiments performed in duplicate, unless otherwise noted. Statistical significance for comparing the \(V_{0.5}\) values of activation and inactivation was determined using an unpaired t-test comparing values of \(V_{0.5}\) calculated from individual experiments. In order to compare the changes in the time constants of inactivation and deactivation, a two-way ANOVA followed by Tukey post hoc analysis was used to compare values at different potentials. Post-hoc tests were run only if F achieved \(P < 0.05\) and there was no significant variance inhomogeneity.
Concentration response curves were fitted to a logistic equation in PRISM. The activation data were fitted with the Boltzmann equation:

\[
G/G_{\text{max}} = \frac{1}{1 + \exp\left(\frac{V_{1/2} - V}{k}\right)},
\]

where \(G/G_{\text{max}}\) is the relative conductance normalized by the maximal conductance, \(V_{1/2}\) is the potential required for half-activation of the current, and \(k\) is the Boltzmann coefficient. Steady-state inactivation data were fitted with the Boltzmann equation:

\[
I = \frac{1}{1 + \exp\left(\frac{V_{1/2} - V}{k}\right)},
\]

where \(V_{1/2}\) and \(k\) are the half-maximum inactivation potential and the slope factor, respectively. The data and statistical analysis comply with the recommendations of the British Journal of Pharmacology on experimental design and analysis in pharmacology (Curtis et al., 2018).

### 2.5 | Materials

All phytocannabinoids used in this study were a kind gift from the University of Sydney’s Lambert Initiative for Cannabinoid Therapeutics. CBD, CBDA, CBDV, CBG, CBGA, CBN, THC, THCA, and THCV were purchased from THCPharm (Frankfurt, Germany) as active pharmaceutical ingredients (APIs). CBC was synthesized by the Lambert Initiative’s Medicinal Chemistry team, and cannabidivaric acid (CBDVA) was synthesized by Professor Michael Kassouo’s team at the University of Sydney. Drugs were kept in concentrated stock solutions in DMSO and stored at \(-30^\circ\)C. Daily dilutions from these stocks were made; the final DMSO concentration in all solutions was 0.1% DMSO, at this concentration, DMSO does not significantly affect the properties of the CaV3 channels. These cannabinoids are not completely soluble in HBSS and 0.1% DMSO at concentrations above about 30 μM. Where possible, we tested drugs up to this almost certainly pharmacologically irrelevant concentration in order to provide as full as possible characterization of the effects of the drugs on the CaV3 channels.

### 2.6 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in the IUPHAR/BPS Guide to PHARMACOLOGY http://www.guidetopharmacology.org and are permanently archived in the Concise Guide to PHARMACOLOGY 2021/22 (Alexander, Christopoulos et al., 2021; Alexander, Mathie et al., 2021).

When referring to phytocannabinoid acids, in all cases, we are referring to the 2-carboxylic acid derivatives, alternatively the “A” acid, for example, THCA-A and CBDA-A.

### 3 | RESULTS

#### 3.1 | The FLIPR measurements of phytocannabinoids on CaV3 channels

In the FLIPR assay of \(\left[\text{Ca}^{2+}\right]_i\), all phytocannabinoids tested modulated the fluorescence signal produced by the activation of CaV3 channels (Table 1). THC and THCV, but not other phytocannabinoids, produced a modest increase in fluorescence when added before the activation of CaV3.1 and CaV3.2, likely due to their actions to shift channel activation to more negative membrane potentials (Mirlohi et al., 2022; Ross et al., 2008). CBGA was the most potent phytocannabinoid, inhibiting CaV3.1 and CaV3.2 with \(pIC_{50}\) values of 6.2 ± 0.1 and 6.4 ± 0.1, respectively; it had much less effect on CaV3.3 (Figure 1a, Tables 1 and S1). The effects of CBGA in the

| Drug (10 μM) | CaV3.1 (% inhibition) | CaV3.2 (% inhibition) | CaV3.3 (% inhibition) |
|-------------|------------------------|------------------------|------------------------|
|             | FLIPR Ephys            | FLIPR Ephys            | FLIPR Ephys            |
| CBGA        | 81 ± 3% 92 ± 2%        | 89 ± 2% 93 ± 1%        | 10.6 ± 1% 91 ± 2%      |
| CBDA        | 81 ± 5% ND             | 22 ± 2% ND             | 6 ± 1% ND              |
| THCA        | 80 ± 2% 96 ± 2%        | 57 ± 8% 53 ± 4%        | 57 ± 5% 43 ± 2%        |
| CBDV        | 74 ± 7% 85 ± 3%        | 62 ± 3% ND             | 11 ± 1% ND             |
| CBDA        | 78 ± 7% ND             | 85 ± 2% ND             | 32 ± 2% ND             |
| CBG         | 78 ± 4% 49 ± 3%        | 37 ± 1% ND             | 10 ± 2% ND             |
| CBN         | 48 ± 2% ND             | 59 ± 2% ND             | 8 ± 1% ND              |
| CBD         | 40 ± 4% 53 ± 3%        | 35 ± 4% 43 ± 4%        | 8 ± 1% 25 ± 3%         |
| CBC         | 36 ± 2% ND             | 26 ± 2% ND             | 9 ± 2% ND              |
| THC         | −49 ± 4% 94 ± 3%       | −93 ± 1% 56 ± 2%       | −13 ± 1% 10 ± 2%       |
| THCV        | −19 ± 2% ND            | −24 ± 4% ND            | −8 ± 3% ND             |

Note: HEK293 cells expressed human recombinant CaV3 channels and were treated with 10 μM of these compounds before \(\left[\text{Ca}^{2+}\right]_i\) levels were measured using the FLIPR assay or \(I_{\text{Ca}}\) were measured using patch clamp. Values show the percentage reduction in fluorescence for cells expressing each channel, or percentage reduction in \(I_{\text{Ca}}\) elicited by a voltage step from −100 mV to −30 mV. Data represent mean ± SEM of \(n = 6\) (Ephys = electrophysiology) (ND = not determined).
FLIPR assay are illustrated in Figure S1A. CBG, the decarboxylation product of CBGA, inhibited CaV3.1 (pIC50 of 5.6 ± 0.1) more effectively than CaV3.2 and CaV3.3 (Figure 1b, Table S1), less potently than CBGA.

CBD (10 μM) showed moderate inhibition of changes in [Ca2+]i following activation of CaV3.1 and CaV3.2 but no inhibition of CaV3.3 (Figures 1c and S1B). CBDA was more potent than CBD; it inhibited CaV3.1 and CaV3.2 with pIC50 values of 5.6 ± 0.1 and 6.0 ± 0.1, respectively (Figure 1d). CBDV, a natural analogue of CBD with a shortened 3 carbon side chain, was also more potent than CBD with pIC50 values of 5.2 ± 0.2 and 5.2 ± 0.1 on CaV3.1 and CaV3.2, respectively (Figures 1e and S1C). CBDVA was also more potent at inhibiting CaV3.1 than CBDV, with a pIC50 of 5.8 ± 0.1, but it produced less inhibition of CaV3.2 than CBDV and no inhibition of CaV3.3 (Table 1). CBC was the least potent inhibitor of CaV3 channels tested (Tables 1 and S1).

In contrast with the results reported above, THC increased [Ca2+]i in cells expressing CaV3.1, 3.2, and 3.3 (by 24 ± 4%), and also produced a change in baseline of 97 ± 3% at 10 μM. THC had modest effects on CaV3.1 and 3.3 (Table 1). In contrast to THC, THCA (pEC50 of 6.0 ± 0.2) inhibited CaV3.1 and reduced the [Ca2+]i increase produced by activation of CaV3.2 and CaV3.3 by approximately 50% at 10 μM (Table 1). CBN, a closer structural analogue of THC, inhibited CaV3.1 with a pIC50 of 5.0 ± 0.2 and also inhibited CaV3.2 by 59 ± 5% at 10 μM but produced no inhibition of CaV3.3 (Table 1).

### 3.2 The effects of CBGA on CaV3 current channels

We next explored CBGA modulation of CaV3 currents using voltage clamp electrophysiology. We first determined the potency of CBGA to modulate channels by superfusion of a single concentration of drug to cells repetitively stepped from −100 to −30 mV. CBGA inhibited CaV3.1, CaV3.2, and CaV3.3 current amplitudes with pIC50 values of 6.47 ± 0.7, 6.0 ± 0.5, and 5.7 ± 0.3, respectively (Figure 2). The inhibitory effects of CBGA on CaV3 channels were only partially reversible over the time course of these experiments (Figure 3a–f).
The effects of CBDV, CBGA, CBG, and CBD on the parameters of steady-state activation and inactivation of CaV3 channels

| Drug    | CaV3 | Change in $V_{0.5}$ | Inactivation |
|---------|------|---------------------|--------------|
|         |      | Activation          | Inactivation |
| CBGA    | 3.1  | $-7 \pm 2^*$        | $-6 \pm 2^*$ |
| CBGA    | 3.2  | $-5 \pm 1^*$        | $-4 \pm 1^*$ |
| CBG     | 3.3  | $1 \pm 0.2$         | $-13 \pm 1^*$|
| CBG     | 3.1  | $1 \pm 0.6$         | $-7 \pm 1^*$ |
| CBVD    | 3.1  | $-3 \pm 1$          | $-8 \pm 3^*$ |
| CBD     | 3.1  | $-1 \pm 0.1$        | $-9 \pm 1^*$ |
| CBD     | 3.2  | $-3 \pm 1$          | $-10 \pm 1^*$|
| CBD     | 3.3  | $-0.7 \pm 0.04$     | $-11 \pm 2^*$|
| Vehicle | 3.1  | $-0.6 \pm 0.1$      | $-1 \pm 0.05$|
| Vehicle | 3.2  | $-1 \pm 0.8$        | $0.5 \pm 0.02$|
| Vehicle | 3.3  | $-1 \pm 0.04$       | $-1 \pm 0.1$ |

Note: HEK293 cells expressing recombinant CaV3 channels were voltage clamped at $-100 \text{ mV}$ and then stepped to potentials above $-75 \text{ mV}$ (activation). To measure inactivation of CaV3, cells were voltage clamped at potentials between $-110$ and $-20 \text{ mV}$ for 3.6 s and then the current measured $-20\text{ mV}$. The membrane potentials at which 50% of channels were activated or inactivated were determined by fitting Boltzmann equations to the data, as outlined in the Methods. The numbers represent the change in these values between control conditions and after 5 min in drug or vehicle (0.1% DMSO), and each value represents the mean ± SEM of six independent experiments.

*P < 0.5 from control (unpaired Student t test).

3.3 | The effects of CBGA and CBG on activation and inactivation of CaV3 channels

We examined the effects of CBGA on CaV3.1 at a range of membrane potentials, as THC has been shown to have complex effects on CaV3 channel gating (Ross et al., 2008). We have previously shown that THC and THCA have similar but not identical effects on CaV3 channel gating (Mirlohi et al., 2022), so in these experiments, we compared CBGA with CBG to see whether this pattern held. Channel activation was examined by holding cells at $-100 \text{ mV}$ and then depolarized from $-75$ to $50 \text{ mV}$ in $5\text{-mV}$ increments. Steady-state channel inactivation was examined by holding cells at $-100 \text{ mV}$ and then stepped every $5\text{ s}$ to test potentials from $-110$ to $-20 \text{ mV}$; $300\text{-nM}$ CBGA increased absolute current amplitude of CaV3.1 for depolarizations between $-75$ to $-55 \text{ mV}$, while current at more positive potentials was strongly inhibited (Figure 4a,b). CBG at $10 \mu\text{M}$ inhibited current amplitude for depolarisation between $-75$ and $50 \text{ mV}$ of Cav3.1 channel but did not increase current amplitude for depolarisation between $-75$ and $-55 \text{ mV}$ (Figure 4c,d). The effects of the EC50 concentration of CBGA on steady-state activation and inactivation of CaV3.1 showed that 300 nM CBGA caused a significant hyperpolarising shift of half activation of CaV3.1 and a significant negative shift in half inactivation of CaV3.1 (Figure 4e, Table 1). The effects of CBG (10 μM) on the CaV3.1 current differed from those of CBGA only in that there was no effect of CBG on channel activation; the membrane potential for CaV3.1 steady-state inactivation was shifted to more hyperpolarized potentials by CBG (Figure 4f, Table 1).

3.4 | CBGA effects on the half activation and inactivation of CaV3.2 and CaV3.3

Similar effects were seen with 1-μM CBGA on the voltage dependence of activation and steady-state inactivation of CaV3.2 (Figure 5a, Table 1). By contrast, CBGA only affected the voltage-dependence of inactivation for CaV3.3, and this was also shifted to negative potentials (Figure 5b, Table 1). CBG did not affect the time to peak or time constant deactivation of CaV3 channels following repolarization (Figure S2A–D).

3.5 | The effects of CBDV and CBD on current amplitude and kinetics of CaV3.1

CBDV inhibited CaV3.1 current amplitude in a concentration dependent manner with a pIC50 of $5.8 \pm 0.06$ and under these conditions CBD (10 μM) also inhibited current amplitude of CaV3.1 by $53 \pm 3\%$ (Figure 6a). CBDV (1 μM) inhibited the current amplitude of Cav3.1 for depolarisation between $-75$ to $50 \text{ mV}$ (Figure 6b). CBD (10 μM) also inhibited the current amplitude of Cav3.1 for all potentials between $-75$ and $50 \text{ mV}$ (Figure 6c). CBDV (1 μM) did not produce a significant change in the membrane potential at which channels were available for the activation however, it produced a significant shift in half inactivation of Cav3.1 (Figure 6d). CBD had no effects on the half activation of Cav3.1 but produced a significant shift of the V0.5 for steady-state inactivation consistent with previous experiments.
CBDV did not affect the time to peak or time constant of deactivation of CaV3.1 (Figure S3A–C). We confirmed that the previously reported lack of effect of CBD on CaV3.2 and CaV3.3 activation and a significant negative shift in channel inactivation was also observed under the present recording conditions, which differed somewhat from those used in previous studies that produced the same result (Ross et al., 2008) (Figure S4A–D).

4 | DISCUSSION

The principle finding of this study is that all 11 phytocannabinoids tested were able to modulate T-type I_Ca to varying degrees in a fluorescent reporter assay of CaV3 channel activation. The compounds had stronger effects on CaV3.1 and CaV3.2 than CaV3.3 in this assay, and while the most common effect was channel inhibition, but CBGA also enhanced activation CaV3 at hyperpolarized potentials, leading to larger Ca^{2+} currents in response to modest membrane depolarization. These findings suggest that while phytocannabinoids may have complex effects on the excitability of cells expressing CaV3, overall they have a common property of producing an inhibition of CaV3 channels. The data also reinforce the potential utility of phytocannabinoids as pharmacological probes for understanding differences between drug binding sites among CaV3 channels as well as on other ion channels.

The acidic phytocannabinoids CBGA, THCA, CBDA, and CBDVA were all more potent than their neutral congeners in the FLIPR assay, and THCA is also more potent than THC at inhibiting CaV3.1 when assessed using patch clamp recordings (Mirlohi et al., 2022). The inhibitory activity of the phytocannabinoid acids is unlikely to be related to changes in pH as the recording solution was strongly buffered, and acidic pH affects T-type I_Ca gating by shifting channel activation to more positive membrane potentials (Delisle & Satin, 2000)—the opposite to what was seen with CBGA, and what has been reported for
FIGURE 4  CBGA and CBG effects on CaV3 channel activation and inactivation. Whole cell patch clamp recordings were made from CaV3 channels stably expressed in HEK 293 cells, 5 min after breaking into cell and then after 5 min in CBGA or CBG. To measure channel activation, cell voltage was clamped at −100 mV then stepped to potential above −75 mV in 5-mV increments. To measure steady-state inactivation, cells were voltage clamped at potentials between −110 and −20 mV in 5-mV increments. (a) Current voltage relationship of HEK293 cells transfected with CaV3.1 in the absence and presence of 300 nM CBGA. (b) Traces from −50- and −20-mV test potentials showed that CBGA potentiated current amplitude of CaV3.1 at lower potentials then inhibited at higher potentials. (c) Current voltage relationship of a representative HEK293 cell transffected with CaV3.1 in the absence and presence of 10-μM CBG. (d) The peak current amplitude is plotted at testing membrane potential of −50 and −20 mV. (e) Steady-state activation and inactivation curves in absence (control) and presence of CBGA and (f) CBG on CaV3.1. Each data point represents the mean ± SEM of 6 cells. Curves are a Boltzmann fit of the data (see method).

FIGURE 5  CBGA effects on CaV3.2 and CaV3.3 activation and inactivation. Whole cell patch clamp recordings were made from CaV3.2 and CaV3.3 channels stably expressed in HEK 293 cells, 5 min after breaking into cell and then after 5 min in CBGA. (a) CBGA did cause significant negative shifts in half activation and inactivation potentials of CaV3.2. (b) The half inactivation potentials of CaV3.3 showed significant negative shifts by CBGA at 1 μM but the half activation of CaV3.3 channels was not changed. Each data point represents the mean ± SEM of 6 cells. Curves were a Boltzmann fit of the data (see Section 2). The effects are reported in Table 1.
Whether less abundant phytocannabinoids are active at CB receptors is a matter of some conjecture, with studies variously finding agonist, antagonist, and no activity for the same compounds, but it seems most likely that 2-carboxylic acid phytocannabinoids do not have the efficacy at CB1 receptors that would be associated with unwanted psychotropic effects (McPartland et al., 2017; Rosenthaler et al., 2014).

The most potent compounds inhibited CaV3-dependent increases in Ca^{2+} with potencies between 300 nM and 3 μM; these potencies are similar to those determined using electrophysiology (Mirlohi et al., 2022; Ross et al., 2008). There is little information about the effects of CBG and CBGA at other voltage gated ion channels, but most minor phytocannabinoids have been reported to inhibit or activate TRP channels (De Petrocellis et al., 2011; Muller et al., 2019). Interestingly, neutral phytocannabinoids are almost always more potent than the corresponding phytocannabinoid acids at modulating TRP channels (De Petrocellis et al., 2011) which is in contrast to the results reported here, where phytocannabinoid acids were more potent in each case.

CBG activates TRPA1 and TRPV1 and inhibits TRPM8 with a potency broadly similar to that reported here for modulation of CaV3 channels (De Petrocellis et al., 2011), while other phytocannabinoids, including THC and CBD, have been found to be more or less potent at TRP channels than CaV3, depending on the assay (Jordt et al., 2004; Neeper et al., 2007). Comparing phytocannabinoid actions on human ion channels expressed in similar systems under similar experimental conditions will provide a better insight into the potential relative contribution of these interactions in more complex systems.

An interesting feature of phytocannabinoid actions is the distinct effects related molecules have on channel gating. At steady state, a concentration of Δ^9-THC at approximately the EC_{50} for inhibition of peak channel produces a negative shift in the membrane potentials at which both CaV3.1 and 3.2 channels open and are inactivated and also slows the deactivation of channel currents on repolarization (Mirlohi et al., 2022; Ross et al., 2008). These effects mean that maximal channel currents evoked from a fixed membrane potential are smaller, but currents resulting from small depolarizations are larger. The effect of

**FIGURE 6** CBDV and CBD effects on CaV3.1 channels. (a) CBDV inhibition on CaV3.1 was concentration dependent. CBD also inhibited CaV3.1 at 10 μM. Whole cell patch clamp recordings were made from human CaV3.1 stably expressed in HEK293. 5 min after breaking into cell and then after 5 min in CBDV or CBD. Cells were voltage clamped at −100 mV and then stepped to potential above −75 mV in 5-mV increments for measuring channel activation. To measure steady-state inactivation, cells were voltage clamped at potential between −110 and −20 mV in 5-mV increments. (b) CBDV and (c) CBD affected current–voltage relationship of the CaV3.1 channel from holding membrane potential of −100 mV. (d) The effects of CBDV and (e) CBD on steady-state activation and inactivation of CaV3.1 channel. Each trace is an example of at least six similar experiments.
Δ²-THC to shift activation of CaV3.1 and CaV3.2 to more negative membrane potentials likely explains the increase in baseline intracellular Ca²⁺ seen following addition of Δ²-THC; Δ²-THC does not change the voltage dependence of activation of CaV3.3 (Mirlohi et al., 2022; Ross et al., 2008) and had no effect on baseline intracellular Ca in CaV3.3 expressing cells. Slower deactivation means more Ca²⁺ entry as cell repolarize to potentials at which CaV3.1/3.2 channels close again. CBGA significantly shifted both activation and inactivation of CaV3.1/3.2 to more negative potentials; CBG, CBD, and CBDV only significantly affected channel inactivation. None of the phytocannabinoids tested in this study altered channel deactivation. We recently reported that THCA had similar effects to THC on CaV3.2 gating; both significantly shifted half activation and inactivation to negative directions and slowed deactivation of CaV3.2 channels (Mirlohi et al., 2022).

It remains to be established whether modulation of T-type Ca²⁺ channels contributes to the therapeutic effects of medicinal Cannabis or pharmaceutical preparations containing THC and/or CBD. Preclinical studies demonstrate the potential involvement of CaV3 channels in a plethora of pathologies, particularly chronic pain states, epilepsy, and gut disorders (Anderson et al., 2019; Scanzi et al., 2016). However, the lack of clinically effective T-type channel Ca²⁺ channel modulators means that the role of these channels in human disease is much less well defined. THC and CBD readily cross the blood brain barrier (Calapai et al., 2020), but in animals at least, phytocannabinoids acids such as THCA, CBDA, CBGA, and CBDVA have lower penetration into the CNS (Anderson et al., 2019). However, in humans, THCA and CBDA are more readily absorbed than THC and CBD when administered as decoction of Cannabis or Cannabis oil (Pellesi et al., 2018; Pérez-Acevedo et al., 2021), and THCA/CBDA have much higher serum concentrations than following equivalent doses of THC/CBD (Pellesi et al., 2018; Pérez-Acevedo et al., 2021). Relative brain levels of phytocannabinoid acids can be increased by careful choice of solvent, and CNS concentrations in the micromolar range can be achieved in animals (Anderson et al., 2019). The serum levels of CBD during chronic oral dosing for paediatric epilepsy are of the order of 600 nM (Contín, Mohamed et al., 2021). It is likely that similar or higher plasma concentrations of THCA and CBD can be readily reached after acute consumption of medicinal cannabis products, and there is little evidence for biotransformation of phytocannabinoid acids to neutral congeners in humans (Anderson et al., 2019; Moreno-Sanz, 2016), with metabolism of THCA possibly paralleling that of THC to produce THCA-COOH (Jung et al., 2007). Thus, concentrations of phytocannabinoids which modulate CaV3 channels in vitro can be achieved in vivo after oral administration of phytocannabinoid acids or plant extracts in which they are enriched, and this may provide a meaningful source of T-channel modulators devoid of at least some of the unwanted effects of THC-containing cannabis preparations. A further area to be explored is the potential interaction at T-type IC₅₀ between Cannabis terpenoids and the phytocannabinoids examined here, as terpenoids alone also modulate these channels (El Alaoui et al., 2017; Gadotti et al., 2021).

This present study shows that phytocannabinoids, alone or in combination, have the potential to exert therapeutic effects on diseases associated with aberrant T-type IC₅₀ activity, without the unwanted psychotropic effects associated with THC-dominant medicinal cannabis. Several studies have demonstrated anticonvulsant effects of cannabis extract preparations (Hussain et al., 2015; Tzadok et al., 2016), but recent studies, including a survey showing the composition and use of unregulated cannabis extracts in the treatment of childhood epilepsy in the Australian community, indicate a significant variability in the minor phytocannabinoid content, making correlation between specific cannabinoid content and effectiveness difficult (Dei Cas et al., 2020; Suraev et al., 2018). However, defining the activities of naturally low abundance phytocannabinoids compounds at human T-type IC₅₀ will contribute to a more complete understanding of their actions in more complex systems and is an essential part of understanding the pharmacology of Cannabis.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge Barry and Joy Lambert for their continued support of the Lambert Initiative for Cannabinoid Therapeutics. Open access publishing facilitated by Macquarie University, as part of the Wiley - Macquarie University agreement via the Council of Australian University Librarians.

AUTHOR CONTRIBUTIONS

SM designed, performed, and analysed the experiments and wrote the manuscript. MJS contributed to the development of the experimental methods. JA and IM were involved in the overall conception of the study. MC and CB contributed to the design and analysis of experiments and writing of manuscript.

CONFLICT OF INTEREST

J.A. is Deputy Academic Director of the Lambert Initiative. He has served as an expert witness in various medicolegal cases involving cannabis and cannabinoids and served as a temporary advisor to the World Health Organization on their review of cannabis and cannabinoids. I.S.M. is Academic Director of the Lambert Initiative and a NHMRC Principal Research Fellow. He is involved in an NHMRC-funded clinical trial using the cannabis extract, Nabiximols (Sativex). He has served as an expert witness in various medicolegal cases involving cannabis and cannabinoids. I.S.M. and J.C.A. are inventors on several patents involving cannabinoid therapeutics. S.M. was supported by an International Research Excellence Scholarship from Macquarie University, C. B was supported by a Macquarie University Research Fellowship. The remaining authors have no conflicts of interest.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the BJP guidelines for Design and Analysis, and as recommended by funding agencies, publishers and other organizations engaged with supporting research.
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How to cite this article: Mirlohi, S., Bladen, C., Santiago, M. J., Arnold, J. C., McGregor, I., & Connor, M. (2022). Inhibition of human recombinant T-type calcium channels by phytocannabinoids in vitro. *British Journal of Pharmacology*, 179(15), 4031–4043. https://doi.org/10.1111/bph.15842

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