Cannabidiol increases gramicidin current in human embryonic kidney cells: An observational study

Mohammad-Reza Ghovanloo [abcd], Samuel J. Goodchild [a], and Peter C. Ruben [b*]

a) Department of Cellular and Molecular Biology, Xenon Pharmaceuticals, Burnaby, BC, Canada.
b) Department of Biomedical Physiology and Kinesiology, Simon Fraser University, Burnaby, BC, Canada.

Current affiliation:
c) Department of Neurology, Center for Neuroscience & Regeneration Research, Yale University School of Medicine, New Haven, CT, USA.
d) Neuro-Rehabilitation Research Center, Veterans Affairs Connecticut Healthcare System, West Haven, CT, USA.

[*] Corresponding Author: Dr. Peter C. Ruben, Department of Biomedical Physiology and Kinesiology, Simon Fraser University, 8888 University Drive, Burnaby, BC, Canada V5A 1S6.
e-mail: pruben@sfu.ca
phone: 778-782-6852
fax: 778-782-3040

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ABSTRACT

Gramicidin is a monomeric protein that is thought to non-selectively conduct cationic currents and water. Linear gramicidin is considered an antibiotic. This function is considered to be mediated by the formation of pores within the lipid membrane, thereby killing bacterial cells. The main non-psychoactive active constituent of the cannabis plant, cannabidiol (CBD), has recently gained interest, and is proposed to possess various potential therapeutic properties, including being an antibiotic. We previously determined that CBD's activity on ion channels could be, in part, mediated by altering membrane biophysical properties, including elasticity. In this study, our goal was to determine the empirical effects of CBD on gramicidin currents in human embryonic kidney (HEK) cells, seeking to infer potential direct compound-protein interactions. Our results indicate that gramicidin, when applied to the extracellular HEK cell membrane, followed by CBD perfusion, increases the gramicidin current.

Keywords: cannabidiol, gramicidin, voltage-clamp, membrane

INTRODUCTION

Linear gramicidins are a family of antibiotics whose function is determined by increasing the cationic permeability of the membrane (Harold & Baarda, 1967; Hladky & Haydon, 1970). Increased permeability is achieved by the formation of bilayer-spanning channels via dimerization of two hemi-channels. Relative to the channels formed by other antibiotics, gramicidin (gA) is well-behaved, and forms channels that are cation selective. Gramicidin channels are also among the best-understood of these types of channels. Atomic-resolution structures have been provided, and a wealth of functional experiments have yielded important insights into gA function (Anon, n.d.; Andersen & Koeppe, 2007). Gramicidin channel monomers that reside in each membrane leaflet must dimerize with monomers in the other leaflet to form a continuous pore. This conformational change is necessary and sufficient for cationic currents to be conducted through gA. The pore diameter is ~4 Å, sufficient to allow the pore to also conduct alkali metals, protons, and water (Hladky & Haydon, 1970, 1972; Finkelstein, 1974; Andersen *et al.*, 2005).

The rate of gramicidin channel dimerization is directly related to membrane stiffness or elasticity (Andersen & Koeppe, 2007; Kapoor *et al.*, 2019). This property has been the foundation of functional assays designed to determine the effects of various compounds on membrane dynamics. For example, compounds that reduce the membrane stiffness or thickness (e.g. detergents) enhance the probability of gramicidin dimerization, which in turn increases the cationic gramicidin signal (Lundbæk *et al.*, 2004; Lundbæk, 2005; Ingólfsson *et al.*, 2010; Kapoor *et al.*, 2019).

Amphiphiles are among the compounds characterized using the gramicidin-based assays (Lundbæk, 2005). Amphiphilic compounds are a set of molecules possessing both lipophilic and hydrophilic properties. These molecules often display non-selective modulatory effects on seemingly unrelated targets, a by-product of amphiphiles modulating membrane elasticity (Lundbæk *et al.*, 2004; Lundbæk, 2005; Kapoor *et al.*, 2019). Modulation is achieved when amphiphiles localize at the solution–bilayer interface, which is made possible by the compounds' polar group residing at the interface with the hydrophobic region, which then inserts into the bilayer core. Partitioning into the lipid bilayer alters membrane elasticity, and changes phase preference and membrane curvature (Lundbæk *et al.*, 2004; Lundbæk, 2005; Kapoor *et al.*, 2019).

One compound with amphiphilic properties is cannabidiol (CBD), the primary non-psychotropic constituent of *Cannabis sativa* (Elsohly, 2007). CBD is a clinically and experimentally substantiated therapeutic compound with efficacy against a variety of conditions, including seizure disorders (for which CBD is FDA-approved), pain, and muscle spasms (Ross *et al.*, 2008; De Petrocellis *et al.*, 2011; Devinsky *et al.*, 2017; Kaplan *et al.*, 2017; Ghovanloo *et al.*, 2018; Iannotti *et al.*, 2019; Fouda *et al.*, 2020). Furthermore, CBD has been suggested to have antibiotic properties (van Klingeren & ten Ham, 1976; Kosgodage *et al.*, 2019). Unlike the psychotropic Δ9-tetrahydracannabinol (THC), CBD has little to no affinity for endocannabinoid receptors (Devane *et al.*, 1988; Lupica *et al.*, 2004). However, many studies have shown that CBD interacts with a wide range of other targets, including a diverse array of ion channels (Ross *et al.*, 2008; De Petrocellis *et al.*, 2011; Kaplan *et al.*, 2017; Ghovanloo *et al.*, 2018, 2021; Fouda *et al.*, 2020; Ghovanloo & Ruben, 2021; Zhang & Bean, 2021). We previously characterized the full inhibitory effects of CBD on voltage-gated sodium channels (Nav) and deciphered the mechanism through which CBD inhibits Nav currents (Ghovanloo *et al.*, 2018, 2021; Sait *et al.*, 2020; Ghovanloo & Ruben, 2021). We further found that an important component of this mechanism involves CBD altering membrane elasticity, which was measured using a gramicidin-based fluorescence assay (GFA) (Ingólfsson *et al.*, 2010; Ghovanloo *et al.*, 2021).

GFA is based on the gramicidin permeability to Tl^+, a quencher of the water-soluble fluorophore 8-aminonaphthalene-1,3,6-trisulfonate (ANTS), encapsulated in large unilamellar vesicles (LUVs) doped with gramicidin. The rate of Tl^+ influx, measured as the rate of fluorescence quench, indicates the time-averaged number of gramicidin channels in the LUV membrane (Ingólfsson *et al.*, 2010). Molecules that alter the thickness and elasticity of the LUV membrane also alter the lipid bilayer contribution to the free energy of dimerization and, thus, the free energy of dimerization (Rusinova *et al.*, 2015). Our previous findings in LUVs suggested that CBD decreases gramicidin signals in that assay (Ghovanloo *et al.*, 2021).

We sought to futher characterize CBD effects on gramaciding gramicidin currents using an electrophysiological HEK cell based assay.In the present study, we investigated the interactions between gramicidin and CBD over short exposures, using voltage-clamped human embryonic kidney (HEK-293) cells in the absence and presence of gramicidin. In this purely observational study, we report that in contrast to the GFA assay, CBD increases the gramicidin current in HEK cells.

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RESULTS

CBD increases gramicidin signal in HEK cells in high extracellular sodium concentrations

Gramicidin channels preferentially conduct cationic (e.g., Na^+ and K^+) currents upon dimerization and pore formation (Harold & Baarda, 1967; Hladky & Haydon, 1970). We measured cationic currents through dimerized gramicidin channels using whole-cell voltage-clamp of untransfected HEK cells in the absence and presence of 26 µM gramicidin applied to the extracellular side of the membrane. First, we measured gramicidin currents in standard high sodium [Na^+=140 mM] extracellular solution using a ramp protocol. We clamped the cell membranes at -80 mV, close to the K^+ equilibrium potential (E_K^+). Then, we hyperpolarized the cells to -120 mV and ramped the voltage to +50 mV, which is close to E_{Na}^+. We show average gramicidin current density from the ratio of current amplitude to the cell membrane capacitance (pA/pF) at -120, -80, 0, and +50 mV (**Figure 1A-D**). Our results indicate that, at negative potentials, gramicidin conducts inward currents and, as the membrane potential becomes more positive, the current becomes outward with the reversal potential (E_{rev}) being close to 0 mV, as would be predicted for a non-specific monovalent cationic channel. We also measured the effects of 1 µM and 10 µM CBD (Ghovanloo *et al.*, 2018), and 10 µM Triton X100 (TX100; as positive control (Lundbæk *et al.*, 2004)) on gramicidin-HEK cells (**Figure 1A-C**). TX100 is a detergent, and has been shown to change membrane elasticity and hence to increase gramicidin current amplitude in equilibrium (Lundbæk *et al.*, 2004). Interestingly, our findings indicate that TX100 reduced the cationic gramicidin currents across all potentials (p<0.05) (**Figure 1C**). CBD had the opposite effect to that of TX100, and slightly increased gramicidin currents at both 1 µM (p<0.05) and 10 µM (p>0.05). Interestingly, although the tendency for CBD to alter gramicidin currents was the same at both concentrations, CBD's effects were more variable at 10 µM than at 1 µM; this variability resulted in lack of statistical significance at 10 µM (**Figure 1C**). We speculate the variability at 10 µM may be due to damage to the HEK cell membrane from both gramicidin and CBD over the timescales of voltage-clamp experiments.

CBD increases gramicidin signal in HEK cells in low extracellular sodium concentrations

The presence of a gramicidin dependent current indicates ion flux across the cell membrane. Gramacidin pores are analogous to puncturing cation-selective holes through the cell

membrane. Gramicidin induced currents in the previous high $[Na^+]$ experiment resulted in a E_{rev} close to 0 mV. This raises the possibility of a potential nonselective leak current component induced by gramicidin, but not carried by gramicidin, as a confounding variable. To ensure that we were recording gramicidin pore currents, we performed the same experiment with lower extracellular sodium $[Na^+=1$ mM]. This experiment resulted in the same overall trends of altered gramicidin currents densities as the high $[Na^+]$ experiment, for both CBD and TX100 (**Figure 2A-D**). As expected, reducing $[Na^+]$ lowered the gramicidin E_{rev} to $\sim$-80 mV (close to E_K^+). These results confirm our results from the high Na^+ experiment and further suggest that, when both Na^+ and K^+ are present at high concentrations, gramicidin permeability is not highly selective for K^+ over Na^+ bringing the gramicidin E_{rev} to $\sim$0 mV. Overall, these results show that CBD increases gramicidin currents during short exposures and suggests, therefore, that CBD alters membrane elasticity or gramicidin channel conductance directly.

DISCUSSION

In our previous study, using a gramicidin-fluorescence assay (GFA), it was determined that CBD has the opposite effect to TX100 and that it decreases the rate of dimerized gramicidin channel formation, and hence a smaller gramicidin current. These findings indicate that CBD is bilateral modifying at the tested concentrations of 1-30 µM (Ghovanloo *et al.*, 2021). In this study, by electrophysiologically measuring K+ and Na+ currents flowing through the gramicidin channel, the opposite result was observed. CBD increased gramicidin currents and decreased TX100 currents suggesting an alternate mechanism of gramacidin interaction with amphiphiles like CBD or TX100 than in the GFA assay.

Although the gramicidin structure does not indicate an obvious direct binding-site for CBD (Anon, n.d.), there is a chance of a direct CBD-gramicidin interaction taking place. Indeed, in almost every report of CBD activity on a given target, a response has been determined, including various ion channels and receptor proteins (Almeida & Devi, 2020; Ghovanloo & Ruben, 2021). Therefore, the opposite result that was observed that might suggest direct CBD and gramicidin interactions that in some way increase the probability of conducting pores in these conditions.

The molecular structure of CBD is composed of two oxygen atoms on both sides of a benzene ring, with the other two ends of the ring having a hydrocarbon tail on one end, and a hydrocarbon ring on the other. These features give the CBD molecule an overall shape that is

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loosely reminiscent of a phospholipid molecule. Phospholipids, in turn, are molecules that specialize in separating various cellular and sub-cellular environments, a function that is dependent on their amphiphilicity. In our previous paper, molecular dynamics (MD) simulations suggested that CBD molecules tend to localize below phospholipid headgroups, but above the tail-end region (Ghovanloo *et al.*, 2021Thus, CBD molecules hovered around carbons ~3-7 of aliphatic chains, as per MD and verified by NMR (Ghovanloo *et al.*, 2021). It is conceivable that interactions between CBD positioned in the leaflet of the HEK membrane may interact with gramicidin hemi channels to impact pore dimerization formation in a way that offset any membrane stiffness affects that inhibit gramicidin currents as we saw in previous GFA studies. In the GFA assay, gramicidin monomers are incubated for 24 hours with liposomes at 13 ˚C to reach equilibration, and then the effect of compound is investigated by measuring fluorescence quenching rates (Ingólfsson *et al.*, 2010). In this study, we measure conventional macroscopic cationic currents in HEK cells using standard voltage-clamp, after the cells were extracellularly perfused with gramicidin monomers over the course of minutes at 27 ˚C. Therefore, the experimental setups between the two studies are fundamentally different, and likely investigate different phenomenon pertaining to gramicidin and CBD interactions.

Our goal in this study was to describe the effects of CBD on HEK cells externally treated with gramicidin. Our results suggest that there may be a direct interaction between CBD and gramicidin but the mechanism by which this potentiates gA currents in HEK cells remains unclear. Further studies will be required for instance, using MD simulations to examine potential for direct interactions.

METHODS

Cell culture

Suspension Human Embryonic Kidney (HEK-293) cells were used for automated patch-clamp experiments. All cells were incubated at 37 ˚C/5% CO_2. All cell culture reagents were purchased from ThermoFisher Scientific, Waltham, MA, unless otherwise noted.

Patch-clamp

Automated patch-clamp recording was performed on untransfected HEK cells. Currents were measured in the whole-cell configuration using a Qube-384 *(Sophion A/S, Copenhagen,*

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Denmark) automated voltage-clamp system. Intracellular solution contained (in mM): 120 KF, 10 NaCl, 2 MgCl$_2$, 10 HEPES, adjusted to pH7.2 with CsOH. The extracellular recording solution for the high sodium experiment contained (in mM): 140 NaCl, 3 KCl, 1 MgCl$_2$, 1.5 CaCl$_2$, 10 HEPES, adjusted to pH7.4 with NaOH. For the low sodium experiment the external solution sodium concentration was lowered to 1 mM with N-methyl-D-glucamine (NMDG) as NaCl replacement. Liquid junction potentials calculated to be ~7 mV were not adjusted for. Currents were low-pass-filtered at 5 kHz and recorded at 25 kHz sampling frequency. Series resistance compensation was applied at 100%. The measurements were obtained at room temperature which corresponds to 27 ± 2 ˚C at the recording chamber. Appropriate filters for cell membrane resistance (typically >500 MΩ) and series resistance (<10 MΩ) were used. Gramicidin was dissolved in 100% DMSO, and the final concentration of 26 µM.

Electrophysiological data analysis

The analysis of raw patch-clamp recordings was performed using the Sophion Analyzer. Graphing and additional analysis was done using the Prism GraphPad (Version 9) software.

Statistics

A one-factor analysis of variance (ANOVA) or t-test were, when appropriate, were used to compare the mean responses. *Post-hoc* tests using the Tukey Kramer adjustment compared the mean responses between channel variants across conditions. A level of significance α=0.05 was used in all overall *post-hoc* tests, and effects with p-values less than 0.05 were considered to be statistically significant. All values are reported as means ± standard error of means (SEM) for *n* recordings/samples.

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M-RG assembled data, performed patch-clamp experiments, data analysis, figure making, wrote manuscript, data interpretation, and assisted to conceiving of experiments. SJG and PCR

conceived the experiments and revised the manuscript critically. All co-authors edited the manuscript.

Competing interests

None. The authors declare that this research was conducted in the absence of competing interests.

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Figure 1 – High sodium voltage-clamp, gramicidin (gA).

(A) Shows the averaged cationic current densities of gramicidin in the presence/absence of CBD at 1 and 10 µM, and TX100 at 10 µM, on the left (in pA/pF, ECS: -120 mV = -13.6 ± 6.3, -80 mV = -10.1 ± 5.0, 0 mV= 5.2 ± 0.5, +50 mV = 30.0 ± 11.0, n = 36; gA: -120 mV = -547.8 ± 66.2, -80 mV = -357 ± 44, 0 mV = 56.5 ± 8.1, +50 mV = 441.1 ± 60.5, n = 47; 1 µM CBD: -120 mV = -820.1 ± 83.2, -80 mV = -543 ± 55.8, 0 mV = 57.7 ± 5.2, +50 mV = 607.4 ± 72.2, n = 56; 10 µM CBD: -120 mV = -687.0 ± 70.3, -80 mV= -452.8 ± 47.4, 0 mV= 57.1 ± 7.1, +50 mV = 649.2 ± 118.8, n = 49; 10 µM TX100: -120 mV = -330.8 ± 43.8 ± , -80 mV = -216.5 ± 30.4, 0 mV= 37.3 ± 4.0, +50 mV = 259.4 ± 32.8, n = 48). The ramp voltage protocol is shown on the right. (B) Shows a cartoon diagram of how gramicidin monomers are thought to dimerize and form channels. (C) Shows quantification of the data shown in (A), stars indicate statistical significance. (D) Shows the associated current traces.

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Figure 2 – Low sodium voltage-clamp.

(A) Shows the averaged cationic current densities of gramicidin in the presence/absence of CBD at 1 and 10 µM, and TX100 at 10 µM (in pA/pF, ECS: -120 mV = -4.1 ± 0.9, -80 mV = -3.5 ± 0.7, 0 mV= 4.1 ± 0.4, +50 mV = 18.8 ± 1.7, n = 33; gA: -120 mV = -67.6 ± 7.7, -80 mV = -23.0 ± 2.9, 0 mV = 189.8 ± 20.6, +50 mV = 437.4 ± 45.6, n = 45; 1 µM CBD: -120 mV = -90.9 ± 9.0, -80 mV = -30.6 ± 2.9, 0 mV = 261.7 ± 28.7, +50 mV = 604.0 ± 64.8, n = 55; 10 µM CBD: -120 mV = -83.6 ± 14.1, -80 mV = -27.8 ± 5.2, 0 mV = 280.8 ± 74.4, +50 mV = 624.9 ± 149.6, n = 60; 10 µM TX100: -120 mV = -26.3 ± 3.5, -80 mV = -9.7 ± 1.3, 0 mV = 74.2 ± 11.3, +50 mV = 182.4 ± 29.0, n = 50). (B) Shows the ramp voltage protocol. (C) Shows quantification of the data shown in (A), stars indicate statistical significance. (D) Shows the associated current traces.
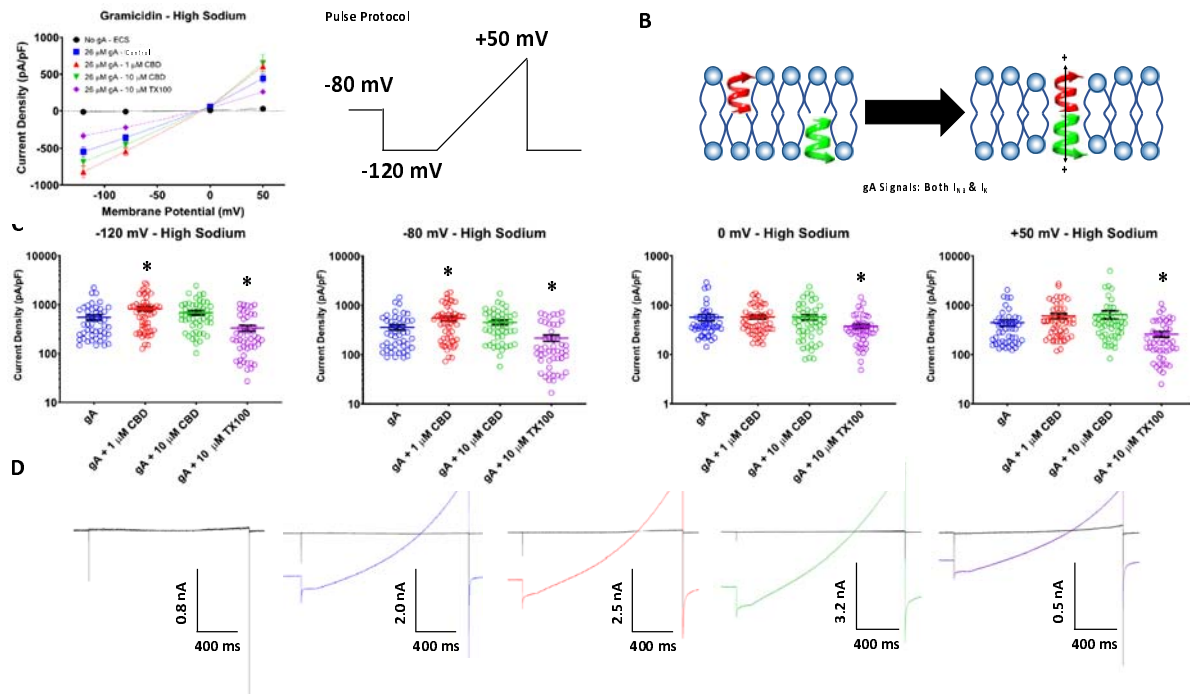
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Figure 1

Figure 2