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Research article

A real time screening assay for cannabinoid CB1 receptor-mediated signaling

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ABSTRACT

The cannabinoid CB1 receptor is expressed throughout the central nervous system where it functions to regulate neurotransmitter release and synaptic plasticity. While the CB1 receptor has been identified as a target for both natural and synthetic cannabinoids, the specific downstream signaling pathways activated by these various ligands have not been fully described. In this study, we developed a real-time membrane potential fluorescent assay for cannabinoids using pituitary AtT20 cells that endogenously express G protein-gated inward rectifier K^+ (GIRK) channels and were stably transfected with the CB1 receptor using a recombinant lentivirus. In whole-cell patch clamp experiments application of the cannabinoid agonist WIN 55,212-2 to AtT20 cells expressing the CB1 receptor (AtT20/CB1) activated GIRK currents that were blocked by $BaCl_2$. WIN 55,212-2 activation of the GIRK channels was associated with a time- and concentration-dependent (EC_{50} = 309 nM) hyperpolarization of the membrane potential in the AtT20/CB1 cells when monitored using a fluorescent membrane potential-sensitive dye. The WIN 55,212-2-induced fluorescent signal was inhibited by pretreatment of the cells with either the GIRK channel blocker tertiapin-Q or the CB1 receptor antagonist SR141716. The cannabinoids displayed a response of WIN 55,212-2 ≈ anandamide (AEA) > CP 55,940 > Δ^9-tetrahydrocannabinol (THC) when maximal concentrations of the four ligands were tested in the assay. Thus, the AtT20/CB1 cell fluorescent assay will provide a straightforward and efficient methodology for examining cannabinoid-stimulated G_i signaling.

1. Introduction

Cannabinoid receptors represent promising targets for the therapeutic management of depression, anxiety, neuropathic pain, obesity, and neurodegenerative disorders (Howlett & Abood, 2017; Kendall & Yudowski, 2016). The cannabinoid receptor family consists of two major G protein-coupled receptors (GPCRs); the cannabinoid type-1 (CB1) and type-2 (CB2) receptors (Howlett & Abood, 2017; Kendall & Yudowski, 2016). The CB1 receptor is widely expressed in the brain and is a receptor for the endogenous eicosanoid anandamide (AEA), the psychoactive plant cannabinoid (−)-*trans*-Δ^9-tetrahydrocannabinol (THC) and synthetic cannabinoids including WIN 55,212-2 and CP 55,940. Although these cannabinoids share the ability to bind to the CB1 receptor, their preference for activating specific G proteins has not been fully defined (Laprairie, Bagher, & Denovan-Wright, 2017; Mallipeddi, Janero, Zvonok, & Makriyannis, 2017). For example, AEA has been reported to strongly activate $G_{i/o}$ and G_q, but to only partially stimulate $G\alpha_s$ (Glass & Northup, 1999; Mukhopadhyay & Howlett, 2005). As a result, AEA decreases cAMP accumulation while stimulating the MAP kinase ERK and protein kinase B (Akt). In contrast, WIN 55, 212-2 appears to stimulate all Gα subtypes, with strong $G_{i/o}$ and G_s activation, and partial G_q activation (Hudson, Hébert, & Kelly, 2010; Mukhopadhyay & Howlett, 2005). Understanding the effects of various cannabinoids on G protein signaling will be necessary for relating cellular actions of the cannabinoids with their therapeutic effects.

G protein-gated inwardly-rectifying K^+ (GIRK) channels are expressed throughout the CNS and peripheral nervous system in areas including the amygdala, ventral tegmental area, cortex, hippocampus, cerebellum, and spinal cord (del Burgo et al., 2008; Marker, Luján, Colón, & Wickman, 2006; Munoz et al., 2016). GIRK channels are composed of the inward rectifier K^+ (Kir) channel subunits Kir3.1, Kir3.2, Kir 3.3 and Kir3.4 (GIRK1, GIRK2, etc.) which are arranged in tetramers. Neuronal GIRK channels are primarily composed of heterotetrameric subunits (Hibino et al., 2010; Lüscher & Slesinger, 2010). GIRK1/2 channels are the most abundant heterotetrameric GIRK subunit arrangement expressed in the CNS and are activated by large number of neuromodulators including opioids, somatostatin, dopamine, acetylcholine and cannabinoids (Hibino et al., 2010; Lüscher & Slesinger, 2010). Binding of these agents to their cognate GPCRs causes the dissociation of the βγ subunits of the pertussis toxin-sensitive $G_{i/o}$

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proteins (Gβγ) which subsequently bind to and activate the GIRK channel (Isomoto, Kondo, & Kurachi, 1997; Logothetis, Kurachi, Galper, Neer, & Clapham, 1987). Once opened, the GIRK channel allows the movement of K^+ out of the cell causing the resting membrane potential to become more negative. Consequently, GIRK channel activation in neurons decreases spontaneous action potential formation and inhibits the release of excitatory neurotransmitters (Hibino et al., 2010; Lüscher & Slesinger, 2010).

Here we report the development of a real-time assay for studying cannabinoid-mediated Gβγ stimulation using a membrane potential-sensitive fluorescent dye. Clonal AtT20 pituitary cells, which endogenously express the GIRK1/2 subunits, were stably transfected with the CB1 receptor (AtT20/CB1) using lentivirus and subsequently studied using the whole-cell arrangement of the patch clamp technique and a fluorescent plate reader. Application of WIN 55,212-2 to the AtT20/CB1 cells caused a time- and concentration-dependent hyperpolarization of the AtT20 cell membrane potential consistent with GIRK channel activation. The fluorescent signal produced by WIN 55,212-2 was inhibited by pretreating the cells with either the GIRK channel blocker tertiapin-Q or the CB1 receptor antagonist SR141716. When tested at maximal concentrations, the cannabinoid ligands WIN 55,212-2, AEA, CP 55,940 and THC were found to display different efficacies and kinetics in activating the GIRK channel. Therefore, the AtT20/CB1 cell fluorescent assay will provide a valuable methodology for determining the ability of various cannabinoid ligands to stimulate G_i and activate GIRK channels.

2. Materials and methods

2.1. AtT20 cell culture and plating

The AtT20 pituitary cell line was obtained from ATCC (AtT-20/D16y-F2, CRL-1795) and grown in DMEM media with 10% fetal bovine serum + Pen-Strep. Cells were plated on uncoated glass coverslips (5000 cells per coverslip) (patch clamp recording) and in poly-L-lysine-coated wells of black 96-well plates (Corning) (30,000 cells per well) (fluorescent measurements). AtT20 cells were stably transfected with lentivirus vectors containing either green fluorescent protein (GFP) or the human cannabinoid type-1 (CB1) receptor (cDNA Resource Center) that were supplied by Dr. Seungjin Shin (Viral Core Facility, University of South Carolina). Cells were stored in an incubator at 37° C (5% O_2/95% CO_2) and used on days 1–3 after plating. Cells expressing GFP were imaged using a Leica DM IL inverted microscope (Vashaw Scientific) and CoolSNAP EZ camera (Photometrics) as described previously (Walsh, 2011).

2.2. CB1 receptor immunoblot analysis

AtT20 cells were harvested in RIPA lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, pH 7.4), and a protease inhibitor cocktail (Pierce Scientific). The cell lysate was pulse sonicated at 2 watts using a model 100 sonic dismembrator (Fisher Scientific) for two periods of 10 s separated by 1 min. The protein content was determined by a Lowry assay. Western blotting was performed as described previously (Piroli et al., 2016) with minor modifications. Briefly, cell lysates were added to loading buffer and incubated for 5 min at 60 °C. Samples were then resolved by SDS/PAGE, transferred to PVDF membranes and blocked for 1 h at room temperature (RT) with 5% non-fat dry milk in wash buffer (20 mM Tris pH 7.4 containing 0.05% Tween 20). Membranes were then incubated at 4 °C with a CB1 receptor Ab (guinea pig L15, a generous gift of Dr. Ken Mackie, University of Indiana) in a 1:2000 dilution in 2% non-fat dry milk in wash buffer. After 3 × 5 min washes with wash buffer, membranes were incubated with a secondary Ab (rabbit anti-guinea pig HRP, Thermofisher) for 1 h at RT, washed 3 × 5 min with wash buffer, developed with Pierce ECL2 and exposed on X-ray films. Membranes were then incubated with 62.5 mM Tris stripping solution (pH 6.8) containing 2% SDS and 0.7% 2-mercapto ethanol for 10 min at 65 °C prior to re-probing with an Ab to actin (loading control) (Santa Cruz Biotechnology).

2.3. GIRK channel fluorescent assay

GIRK channel activation was monitored in the 96-well plates by fluorescently recording the cell membrane potential (MP) (Günther, Culler, & Schulz, 2016; Walsh, 2011) and has been described in detail (Vazquez, Dunn, & Walsh, 2012). For the MP measurements, cells were incubated for 30 min in normal buffer solution consisting of; 132 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM dextrose, 5 mM HEPES, pH 7.4 (with NaOH), with a MP-sensitive fluorescent dye (FLIPR Membrane Potential kit RED; Molecular Devices). Prior to the fluorescent measurements, the cells were loaded with dye in buffer solution containing 1 mM KCl and incubated for an additional 5 min. Fluorescent signals were recorded using a Synergy2 microplate reader (Biotek) at 28° C (Walsh, 2011). Cannabinoid ligands were dissolved in DMSO (WIN 55,212–2, AEA & CP 55,940) or alcohol (THC) at stock concentrations of 10–33 mM and diluted to various concentrations in 1 mM KCl buffer solution containing the dye. The cannabinoids or control solution (10 or 20 µl) were added to each well (total volume = 110 or 220 µl) at time zero using an injector. Data points were collected at 5 s intervals over a 250 s sampling period at excitation and emission wavelengths of 520 and 560 nm, respectively. For each experiment, the control fluorescent signal (usually the first two wells in each row of the plate) was subtracted from the signal obtained in the presence of the cannabinoids. The concentration versus response curve for WIN 55,212–2 was determined by fitting the data with the curve: $Max/(1 + ([drug]/EC_{50})^k)$, where the EC_{50} is the concentration producing a 50% increase in the maximal response and k is the slope factor. Where appropriate, means ± SE for the ligands were determined and the statistical significance estimated using Student's *t*-test for unpaired observations.

2.4. Patch clamp recording

The patch clamp method (Hamill, Marty, Neher, Sakmann, & Sigworth, 1981) was used to record the whole-cell, GIRK currents using L/M EPC-7 (Adams & List Associates) and Axopatch 200 (Molecular Devices) amplifiers (Walsh, 2011). Pipettes were made from borosilicate glass capillaries (World Precision Instruments) and had resistances of 2–3 Mohms when filled with internal solution. All experiments were conducted on isolated, non-coupled cells at room temperature (22–24 °C). GIRK currents were measured in external solution consisting of; 95 mM NaCl, 50 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM dextrose, 5 mM HEPES, pH 7.4 (with NaOH). High external K^+ was used in order to increase the driving force for K^+ movement through the GIRK channel and allow the measurements on inward GIRK currents. The internal solution consisted of; 50 mM KCl, 60 mM K^+-Glutamate, 2 mM $MgCl_2$, 1 mM EGTA, 2 mM ATP, 0.1 mM GTP, 10 mM HEPES, pH 7.3 (with KOH). Following the measurement of the cell background current, GIRK channels were activated by the addition of 5 µM WIN 55,212-2 using a perfusion system. In each experiment the GIRK current was defined as the $BaCl_2$-sensitive current (Walsh, 2011).

2.5. Drugs and chemicals

WIN 55,212-2, anandamide (AEA), CP 55,940 and SR141716 were purchased from Cayman Chemical. Δ^9-tetrahydrocannabidol (THC) was gift from Dr. Mitzi Nagarkatti (University of South Carolina). Tertiapin-Q was purchased from Alomone Laboratories (Jerusalem, Israel).