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Cannabinoid receptor-mediated translocation of NO-sensitive guanylyl cyclase and production of cyclic GMP in neuronal cells

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Abstract

Cannabinoid agonists regulate NO and cyclic AMP production in N18TG2 neuroblastoma cells, leading to the hypothesis that neuronal cyclic GMP production could be regulated by CB_1 cannabinoid receptors. NO (nitric oxide)-sensitive guanylyl cyclase (GC) is a heterodimeric cytosolic protein that mediates the down-stream effects of NO. Genes of proteins in the cyclic GMP pathway (α_1, α_2, and β_1 subunits of NO-sensitive GC and PKG1, but not PKG2) were expressed in N18TG2 cells, as was the CB_1 but not the CB_2 cannabinoid receptor. Stimulation of N18TG2 cells by cannabinoid agonists CP55940 and WIN55212-2 increased cyclic GMP levels in an ODQ-sensitive manner. GC-β_1 in membrane fractions was increased after 5 or 20 min stimulation, and was significantly depleted in the cytosol by 1 h. The cytosolic pool of GC-β_1 was replenished after 48 h of continued cannabinoid drug treatment. Translocation of GC-β_1 from the cytosol was blocked by the CB_1 antagonist rimonabant (SR141716) and by the Gi/o inactivator pertussis toxin, indicating that the CB_1 receptor and Gi/o proteins are required for translocation. Long-term treatment with rimonabant or pertussis toxin reduced the amount of GC-β_1 in the cytosolic pool. We conclude that CB_1 receptors stimulate cyclic GMP production and that intracellular translocation of GC from cytosol to the membranes is intrinsic to the mechanism and may be a tonically active or endocannabinoid-regulated process.

Keywords: CB_1 cannabinoid receptor; CP55940; cyclic GMP; Gene expression; G-protein coupled receptors; G-proteins; NO-sensitive guanylyl cyclase; Protein translocation; Real-time polymerase chain reaction; Rimonabant (SR141716); WIN55212-2

1. Introduction

CB_1 cannabinoid receptors are found in great abundance in brain and peripheral neuronal systems (for review, see Howlett et al., 2002a; Pazos et al., 2005). These G-protein coupled receptors mediate the response to endocannabinoid eicosanoid mediators, particularly anandamide and 2-arachidonoylglycerol, in neurons and other cells throughout the body (Fowler et al., 2005; Bisogno et al., 2005). Agonist drugs for the CB_1 receptor include CP55940 and WIN55212-2, and antagonist drugs include rimonabant, also known as SR141716 (Howlett et al., 2002a; Beardsley and Thomas, 2005). CB_1 receptors are found predominantly on presynaptic locations, and are important in the regulation of neurotransmitter release (Schlicker and Kathmann, 2001; Diana and Marty, 2004). CB_1 receptors block voltage-gated Ca^{2+} channels leading to reduced probability of transmitter release, and also inhibit cyclic AMP production and thereby decrease protein kinase (PK) A-mediated regulation of K^+ channels leading to hyperpolarization (reviewed in Childers and Deadwyler, 1996; Howlett et al., 2004).

We determined that stimulation of CB_1 receptors increased NO (nitric oxide) production in the neuronal N18TG2 cell model (Mukhopadhyay et al., 2002). One target for NO is

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the NO-sensitive guanylyl cyclase (GC), also known as "soluble" GC, which exists as a heterodimer of α and β subunits. NO binding to NO-sensitive GC catalyzes the conversion of GTP to the second messenger cyclic GMP, which regulates PKG to phosphorylate target proteins. In cDNA microarray studies, we found that GC-β1 mRNA was expressed in N18TG2 cells and could be increased after 48-h exposure to cannabinoid agonists (Howlett et al., 2002b). In determining the cellular regulation of the protein content, it was discovered that stimulation of the CB_1 receptor resulted in a diminution of GC in the soluble fraction. The present studies report the translocation from the soluble fraction to the membranes concurrent with cyclic GMP production, and the requirements for stimulation of the CB_1 receptor and Gi/o proteins for translocation and tonic regulation of GC levels.

2. Materials and methods

2.1. Materials

The reagents were purchased from Sigma Chemical Company (St. Louis, MO), unless otherwise stated. CP55940 ((−)-*cis*-3R-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4*R*-3(3-hydroxypropyl)-1*R* cyclohexanol) and rimonabant (*N*-(piperidin-1yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-*H*-pyrazole-3-carboxamide) were provided by the National Institute of Drug Abuse drug supply program. WIN55212-2 (*R*(+)-[2,3-dihydro-5-methyl-3[(morpholinyl)methyl]pyrrolo[1,2,3-de]-1,4-benzoxazinyl]-(*l*-naphthalenyl) methanone mesylate), rolipram and 1-isobutyl-3-methyl-xanthine (IBMX) were from Sigma, and acrylamide, *N*,*N*,*N*′,*N*-tetramethylethylene diamine, sodium dodecylsulfate (SDS) and polyvinylidine difluoride membranes were from BioRad Laboratories, Inc. (Hercules, CA). 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) and GC antibody (catalog # 371712) were from Calbiochem (La Jolla, CA). GC-β_1 subunit (soluble) polyclonal antibody (rabbit) (catalog # 160897) was purchased from Cayman Chemical (Ann Arbor, MI). Goat anti-rabbit IgG horseradish peroxidase (catalog # ALI3404) was from Biosource International (Camarillo, CA). Goat serum was from Gibco Life Technologies (Gaithersburg, MD). Rainbow molecular weight markers, Enhanced Chemiluminescence detection kit, and high performance autoradiography film (Hyperfilm) were from Amersham Bioscience (Piscataway, NJ). The RNeasy Mini purification kit was from Qiagen (Valencia, CA). TaqMan Universal PCR Master Mix and Assays-on-Demand Gene Expression Assay mixes specific for mouse 18S, CB_1 and CB_2 receptors, α1, α2, β1, and β2 subunits of GC, and PKG1 and PKG2 were from Applied Biosystems, Inc. (Foster City, CA).

2.2. Cell culture

N18TG2 neuroblastoma cells (passage numbers 25–50) were maintained in 75 cm^2 flasks at 37 °C in Dulbecco's modified Eagle's medium:Ham's F-12 (1:1) with GlutaMax, sodium bicarbonate, and pyridoxine–HCl, supplemented with penicillin (100 units/ml) and streptomycin (100 μg/ml) (Gibco Life Technologies, Gaithersburg, MD) and 10% heat-inactivated bovine serum (JHR Bioscience, Lenexa, KS). Drug stocks were stored at −20 °C as 10 mM solutions in ethanol. An aliquot was air-dried under sterile conditions in trimethylsilyl-coated glass test-tubes and was taken up in 100 volumes of 5 mg/ml fatty acid-free bovine serum albumin. From this, 100 μl was added to flasks containing 10 ml of media, providing a final concentration of 1 μM CP 55940 or WIN55212-2, or a drug-free vehicle. Where indicated, N18TG2 cells were pretreated with the CB_1 antagonist rimonabant (1 μM) for 30 min prior to addition of agonists, or at the times indicated before harvesting. Pertussis toxin (100 ng/ml final concentration) (BIOMOL, Plymouth Meeting, PA) was added to flasks containing fresh media 16 h before addition of agonists, or at the times indicated before harvesting.

2.3. Gene expression determinations

Total RNA was extracted and purified from N18TG2 cells according to the instructions provided with the Qiagen RNeasy Mini Kit and purity was determined spectrophotometrically using the 260/280 ratio. Total RNA (1 μg) was reversed transcribed into cDNA using random hexamers according to the methods described in the Applied Biosystems cDNA Archive Kit. Real time PCR reactions were performed in a 25 μl reaction volume using TaqMan Universal PCR Master Mix and appropriate primer and probes according to the Applied Biosystems, Inc. instructions using a 7500 Real-time PCR System. Ribosomal 18S RNA was the reference standard gene, and relative quantitation was determined using the Delta Delta Ct method (Livak and Schmittgen, 2001).

2.4. Cyclic GMP determination

N18TG2 cells grown to confluence on 96-well culture plates were washed with physiological saline solution (PSS) (150 mM NaCl, 5.4 mM KCl, 1.18 mM $MgSO_4$, 1 mM $CaCl_2$; 6 mM $NaHCO_3$, 5.5 mM D-glucose), and incubated for 5 min with PSS containing 0.1 mM IBMX and 0.1 mM rolipram prior to addition of drugs. When ODQ was included, the inhibitor was added during the 5 min preincubation. Cannabinoid drugs were added for 5 min or 20 min at 37 °C, and the incubation was stopped by aspiration of the media and freezing the plate at −80 °C. Cellular cyclic GMP was determined using the Enzyme-immunoassay Biotrak System Kit (Amersham Biosciences, Piscataway, NJ). Briefly, lysis reagent was added to the wells, and aliquots were acetylated prior to immunoassay using cyclic GMP–horseradish peroxidase conjugate and substrate according to the instructions, and the optical density was read at 450 nm using a VICTOR^3 multiplate reader (Perkin Elmer, Wellesley, MA). The standard curve was analyzed and fit, and unknown samples were determined using Prism IV (Graphpad, San Diego, CA).

2.5. Western blot analysis

After treatment, N18TG2 cells were harvested with PBS-EDTA (2.7 mM KCl, 138 mM NaCl, 10.4 mM glucose, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, 0.625 mM EDTA, pH 7.4). The cells were suspended in cold TME buffer (20 mM Tris–Cl, pH 7.4, 3 mM $MgCl_2$, 1 mM Na EDTA) with a protease inhibitor cocktail (set III, Cat. No. 59134, Calbiochem, La Jolla, CA) having a broad ability to inhibit aspartic, cysteine, serine, and aminopeptidases. The cells were allowed to swell in the hypotonic solution (15 min), and then were homogenized with a glass–glass homogenizer and sedimented at 1000 × *g* at 4 °C for 10 min. The pellet, which contained the nuclear and cellular debris, was discarded and the supernatant was sedimented at 100,000 × *g* for 30 min at 4 °C, and the membranes were resuspended in 1/20 the cytosol volume. The supernatants and membrane pellets resuspended in cold 50 mM Tris–Cl buffer pH 7.4 were stored in aliquots at −80 °C. Rat forebrain cytosolic fractions were prepared from frozen whole rat brains purchased from Pel-Freeze (Rogers, AK). The brains were thawed in ice-cold SME solution (320 mM sucrose; 5 mM $MgCl_2$; 2 mM Tris–EDTA). The brain tissue was homogenized in a glass–glass homogenizer in 2 ml of SME per gram of tissue, and sedimented at 1000 × *g* at 4 °C for 10 min to remove cellular and nuclear debris. The supernatant was saved and pellet resuspended in 1 ml of SME for a second sedimentation. The supernatants were combined and sedimented at 39,000 × *g* at 4 °C for 25 min. The cytosolic fractions were stored in aliquots at −80 °C until use. The protein concentrations were determined using the Coomassie dye binding method (Bradford, 1976).

N18TG2 fractions were taken up in Laemmli's sample buffer with 1 mM dithiothreitol and heated at 60 °C for 10 min. Unless otherwise indicated, equal amounts of protein (7 or 12 μg) were loaded per lane and SDS-10% polyacrylamide gel electrophoresis (PAGE) was run at 50 V for 30 min and then 120 V for 80 min. The proteins were transferred to polyvinylidine difluoride membranes in Towbin's buffer (24 mM Tris Base, 192 mM glycine and 20% methanol; pH 8.3) for 1 h in the cold at 95 volts using a BioRad Trans-Blot Cell with an ice pack. Blots were rinsed three times (5 min each) with Tris-buffered saline (TBS) (20 mM Tris–Cl, pH 7.4, 137 mM NaCl) and incubated with blocking solution (5% nonfat dry milk, 5% normal goat