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Cannabinoid signaling in rat cerebellar granule cells: G-protein activation, inhibition of glutamate release and endogenous cannabinoids

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Abstract

Previous studies have indicated that cannabinoids inhibit presynaptic neurotransmitter release in brain through CB_1 receptors. To examine this issue in a primary neuronal culture system, rat cerebellar granule cells (CGCs) were prepared. [^{35}S]GTPγS binding assays in saponin-permeabilized CGCs showed that G-protein activation by the CB_1 agonist, WIN55212-2, and adenosine A_1 agonist, phenylisopropyladenosine, was maximal during the second week in culture. Δ^9-tetrahydrocannabinol stimulated [^{35}S]GTPγS binding to a lesser degree than WIN55212-2, and the antagonists SR141716A and AM281 acted as inverse agonists in intact CGCs, but not in CGC membrane preparations. Ten micromolar WIN55212-2 and Δ^9-tetrahydrocannabinol decreased depolarization-evoked efflux of [^{3}H]-D-aspartate from CGCs by 32% and 13%, respectively. SR141716A and AM281 increased [^{3}H]-D-aspartate release by 28%. The fatty acid amide hydrolase (FAAH) inhibitor phenylmethylsulfonyl fluoride (PMSF) and the anandamide uptake inhibitor AM404 inhibited transmitter release, implying that the antagonist effects were mediated by blockade of endocannabinoid activity. Levels of endocannabinoids (both anandamide and 2-arachidonyl glycerol [2-AG]) in extracts of the cells and cell incubation buffer were increased by PMSF pre-treatment. Depolarization with KCl significantly decreased the amount of anandamide and 2-AG in PMSF-treated CGCs. These results suggest that endogenous cannabinoids inhibit neurotransmitter release in CGCs, which may also release endocannabioids upon neural stimulation.

Keywords: Neurotransmitter release; Cerebellar granule cell; Cannabinoid; CB_1 receptor; Efficacy; G-protein; Guanosine-5′-*O*-(3-thiotriphosphate); Arachidonyl ethanolamide; Anandamide; 2-Arachidonoyl glycerol

1. Introduction

The term "cannabinoid" originally referred to a family of compounds structurally related to Δ^9-tetrahydrocannabinol, the biologically active constituent from the plant *Cannabis sativa* (Gaoni and Mechoulam, 1964). Since the discovery of specific cannabinoid receptors, the term has come to include all compounds that interact with cannabinoid receptors regardless of their basic structure. Two types of cannabinoid receptors, CB_1 (Matsuda et al., 1990) and CB_2 (Munro et al., 1993) have been cloned, and both belong to the superfamily of G-protein-coupled receptors.

Activation of G-proteins by cannabinoid agonists involves receptor-mediated release of guanosine diphosphate (GDP) from an inactive G-protein alpha subunit, which allows guanosine triphosphate (GTP) to

Abbreviations: 2-AG, 2-arachidonoyl glycerol; CGC, cerebellar granule cell; FAAH, fatty acid amide hydrolase; GDP, guanosine diphosphate; GTPγS, guanosine-5′-*O*-(3-thiotriphosphate); PMSF, phenylmethylsulfonyl fluoride

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bind to and activate the alpha subunit and promotes dissociation of the beta–gamma subunit complex. Activation of G_i and G_o type G-proteins by cannabinoid agonists (Prather et al., 2000) has been measured by agonist-stimulated binding of the non-hydrolyzable GTP analog [^{35}S]GTPγS to brain membranes or slide-mounted brain sections (Breivogel et al., 1997b; Sim et al., 1996b). Detection of cannabinoid-stimulated [^{35}S]GTPγS binding in primary neuronal cultures is more difficult because of the relatively low signal. In the present study, we demonstrate cannabinoid agonist-stimulated [^{35}S]GTPγS binding to intact cultured cells by first permeabilizing the cell membranes with saponin to allow [^{35}S]GTPγS access to intracellular G-proteins.

CB_1 receptors are found in the highest density in the globus pallidus, substantia nigra, the entopeduncular nucleus and the molecular layer of cerebellar cortex (Herkenham et al., 1991). The majority, if not all, of the cannabinoid receptors in cerebellum are located on axon terminals of cerebellar granule cells (CGCs; Tsou et al., 1998), glutamatergic neurons that project to cerebellar Purkinje cells. The majority of electrophysiological studies of neurons in culture (Chan et al., 1998; Shen et al., 1996; Shen and Thayer, 1998), in brain slices (Gifford and Ashby, 1996; Robbe et al., 2001; Schlicker et al., 1997), and in vivo (Gessa et al., 1997, 1998; Levenes et al., 1998; Miller and Walker, 1995; Sañudo-Peña and Walker, 1997; Tersigni and Rosenberg, 1996) have indicated that cannabinoids reduce neurotransmitter release. However, there have also been results from other studies that showed transmitter levels may be increased by cannabinoids. In pallidal slices GABA reuptake was inhibited with no effect on GABA release (Maneuf et al., 1996a,b). Another study found that WIN55212-2 increased extracellular glutamate levels in rat cortex or cultured cortical neurons (Ferraro et al., 2001). These studies have suggested that cannabinoids have multiple effects on neurotransmitter levels depending on the brain region and the neurotransmitter in question.

More recently, endogenous cannabinoids have been described as retrograde neuromodulators in several brain areas, meaning that excitation of a postsynaptic neuron increases intracellular Ca^{2+} levels, which induces endocannabinoid synthesis and release. The endocannabinoids travel retrograde to the presynaptic neuron terminal to activate cannabinoid receptors and inhibit or modulate subsequent neurotransmitter release (Diana et al., 2002; Kreitzer and Regehr, 2001a,b; Ohno-Shosaku et al., 2002; Takahashi and Linden, 2000; Wilson and Nicoll, 2001). Previous studies from our laboratory (Pacheco et al., 1993) have shown that cannabinoid agonists inhibit adenylyl cyclase activity in primary cultures of rat CGCs. One of the major goals of the present study was to determine whether cannabinoids decrease glutamate release from CGCs by directly measuring depolarization-induced efflux of [^{3}H]-D-aspartate. We also investigate the possible roles of two endocannabinoids, anandamide and 2-AG, in CGC signaling. The majority of this work was presented at the International Cannabinoid Research Society 1999 Symposium on the Cannabinoids (Breivogel et al., 1999).

2. Materials and methods

2.1. Materials

Female Sprague–Dawley rats and pups were purchased from Zivic Miller Laboratories, Inc. (Zelienople, PA). [^{35}S]guanosine-5′-*O*-(3-thiotriphosphate) ([^{35}S]GTPγS) (1250 Ci/mmol), and [D-2,3-^{3}H] aspartic acid (18.0 Ci/mmol) were purchased from New England Nuclear Corp. (Boston, MA). Δ^9-tetrahydrocannabinol was provided by NIDA/Research Triangle Institute (Research Triangle Park, NC). WIN55212-2 was purchased from Research Biochemicals International (RBI; Natick, MA). SR141716A was a generous gift from Dr Francis Barth at Sanofi Recherché (Montpellier, France). AM281 and AM404 were purchased from Tocris (Ballwin, MO). All ligands were dissolved in ethanol or dimethylsulfoxide (DMSO) at 10–30 mM; final concentrations of the vehicles in the assays were no more than 0.1%. GTP, GDP and GTPγS were purchased from Boehringer Mannheim (New York, NY). Synthetic standards of anandamide were obtained from RBI, [$^2H_8$]-anandamide and 2-arachidonoyl glycerol (2-AG) from Cayman Chemical Company (Ann Arbor, MI). All other reagent grade or tissue culture grade chemicals and enzymes were obtained from Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA).

2.2. Preparation of cerebellar granule cell primary cultures

CGCs were cultured as previously described (Lasher and Zagon, 1972; Pacheco et al., 1993). Animals were treated in accordance with the National Institutes of Health guide for the care and use of Laboratory animals. Eight-day-old rat pups were sacrificed by injection of Nembutal Sodium Solution (Abbott Laboratories, North Chicago, IL) and brains were removed and placed in neuronal saline with 1% antibiotic antimycotic solution (10,000 units/ml penicillin, 10 mg/ml streptomycin and 25 μg/ml amphotericin, Sigma Chemical Co.). Cerebella were removed, minced and incubated with 0.25% trypsin for 10 min and 160 μg DNase for 5 min at 37 °C. The reactions were terminated by the addition of 10 ml growth media

(DMEM with 25 mM KCl, 1% antibiotic antimycotic solution and 10% fetal bovine serum, GibcoBRL Life Technologies, Grand Island, NY), then the suspension was centrifuged at 60–70×*g* for 3 min. The supernatant was discarded, and the cells were resuspended and triturated to homogeneity in 10 ml growth media using a glass pipet, then passed through a 40 μm sterile nylon cell strainer (PALL, Gelman Sciences, Ann Arbor, MI). Cells were brought up to final volume (~13 ml/pup) in growth media, and plated at 1 ml/well into poly-L-lysine coated 12-well cell culture plates (Corning Incorporated, Corning, NY). After one day in culture and every three subsequent days, the media was replaced with growth media containing 10 μM cytosine arabinoside (ara-C). Except when noted, all experiments were performed after 7–11 days in culture. One day prior to assaying each plate, the growth media was replaced with media lacking fetal calf serum.

2.3. [^{35}S]GTPγS binding

[^{35}S]GTPγS binding to whole cells was performed using saponin permeabilization. Cells were rinsed two times for 5 min at 37 °C with 0.5 ml/well assay buffer (50 mM Tris–HCl, 3 mM $MgCl_2$, 0.2 mM EGTA, 100 mM NaCl, pH 7.4), then incubated for 2 min at room temperature (r.t.) in 0.5 ml/well saponin solution (140 mM potassium glutamate–HCl, pH 6.8, with 1 mg/ml ATP and 0.1 mg/ml saponin) to permeabilize the cell membranes. In some assays the incubation time was varied and some wells of cells were treated with the solution minus saponin as controls. In other assays, 50 μM phenylmethylsulfonyl fluoride (PMSF) was added to the saponin solution to irreversibly inhibit amidase enzymes in the cells, including fatty acid amide hydrolase (FAAH), the enzyme that degrades endocannabinoids anandamide and 2-AG. Cells were rinsed again two times with assay buffer, then preincubated in assay buffer for 10 min at 37 °C with 100 μM GDP and 0.004 units/ml adenosine deaminase (220 units/mg protein, Sigma Chemical Co.) in the presence and absence of various ligands. The assay was initiated by the addition of 0.1 nM [^{35}S]GTPγS and incubated for 1 h; non-specific binding was determined in the presence of 25 μM unlabeled GTPγS. The assay was terminated by transferring the cells and buffer to glass test tubes on ice. Aliquots of each sample were transferred to another set of test tubes to be assayed for protein content (Bradford, 1976) before the filtration of the cells under vacuum through Whatman GF/B glass fiber filters, followed by three washes with cold Tris–HCl buffer, pH 7.4. Bound radioactivity was determined by liquid scintillation spectrophotometry at 95% efficiency for [^{35}S] after overnight extraction of the filters in 4 ml ScintiSafe Econo 1 scintillation fluid (Fisher Scientific).

[^{35}S]GTPγS binding in membrane homogenates from cultured CGCs was performed with WIN 55212-2 as an agonist, as previously described for mu and delta opioid receptors in cultured neuroblastoma cells (Breivogel et al., 1997a).

2.4. [^{3}H]-D-aspartic acid release from CGCs

CGCs were rinsed two times for 5 min at 37 °C with 1 ml/well Krebs–Ringer buffer (128 NaCl, 5 mM KCl, 2.7 mM $CaCl_2$, 1 mM Na_2HPO_4, 1.2 mM $MgSO_4$, 20 mM HEPES pH 7.4, 10 mM glucose and 1 mg/ml bovine serum albumin), and were then incubated for 15 min at 37 °C in 1 ml/well Krebs–Ringer buffer with 1 μCi/well [^{3}H]-D-aspartic acid. In some assays, 50 μM of the amidase inhibitor, PMSF was added to some wells with the [^{3}H]-D-aspartic acid. Cells were rinsed again three times for 3 min at 37 °C with 1 ml/well Krebs–Ringer buffer, then plates were loaded into the Superfusion-12 apparatus (Brandel, Gaithersburg, MD), which had been rinsed with Krebs–Ringer buffer and pre-warmed to 37 °C. Cells were rinsed in the apparatus for 10 min at 0.3 ml/min with Krebs–Ringer buffer with or without cannabinoid drugs before collection (3 min intervals) was initiated. After 12 min, a high [KCl] version of Krebs–Ringers buffer (Krebs–Ringer buffer with 33 mM NaCl and 100 mM KCl) containing identical drug concentrations was substituted for the Krebs–Ringer buffer, and collection was continued for an additional 33 or 48 min. Each condition was determined in triplicate including basal high-[KCl]-induced release which was determined in the absence of cannabinoid ligands. The amount of [^{3}H]-D-aspartic acid collected was determined by liquid scintillation spectrophotometry at 45% efficiency for [^{3}H] after addition of 4 ml Ecolite scintillation fluid (Fisher Scientific).

2.5. Lipid extraction of CGCs

CGCs were rinsed three times for 5 min at 37 °C with 1 ml/well Krebs–Ringer buffer (128 mM NaCl, 5 mM KCl, 2.7 mM $CaCl_2$, 1 mM Na_2HPO_4, 1.2 mM $MgSO_4$, 20 mM HEPES pH 7.4, 10 mM glucose and 1 mg/ml bovine serum albumin), then incubated for 10 min with 50 μM PMSF before an additional rinse. Some assays omitted the PMSF pre-treatment to determine the effectiveness of the treatment. Cells were incubated for 60 min in 0.5 ml/well Krebs–Ringer buffer or high-[KCl] Krebs–Ringer buffer (to depolarize the neurons). Each condition was performed in quadruplicate. Incubations were terminated by the transfer of the buffer to glass tubes for extraction. [$^2H_8$]-anandamide standard (500 fmol) was added to each tube along with two volumes 2:1 (v/v) chloroform:methanol, and each was shaken vigorously to mix the aqueous and organic

phases which were then allowed to separate three times. The aqueous phase was removed by pipet, then the organic phase was evaporated in a Speed-vac and stored at −80 °C until analysis. Methanol (500 μl) was added to each well of cells, and 500 fmol of [$^2H_8$]-anandamide standard was added to one well of cells from each condition (total of six wells/condition) before incubating for 15 min at r.t. The methanol was removed and pooled from six wells that were treated under the same conditions. The methanol extract was centrifuged at $30,000\times g$ for 10 min and the supernatant evaporated in a Speed-vac and stored at −80 °C until analysis. The pellet was stored to be combined with remaining cell material. NaOH (500 μl, 0.1 N) was added to each well of cells and allowed to stand overnight before dissolved cell material was added to the pellet described above. Combined cell material was assayed for protein content for normalization of the amount of endogenous cannabinoids.

2.6. Liquid chromatography/mass spectrometry (LC/MS) analysis of CGC extracts

Samples were reconstituted in 100 μl methanol and capped under nitrogen. The samples were vortexed for 1–2 min and microcentrifuged for 6 min. The supernantant was applied to the LC/MS for analysis of endocannabinoids. LC/MS (Agilent 1100 series, Wilmington, DE) was used for the analysis of CGC extracts for anandamide, 2-AG and the internal standard [$^2H_8$]-anandamide. Samples were applied to the LC in 30 μl volumes. The parameters for the LC/MS employed were similar to those reported previously (Walker et al., 1999; Huang et al., 1999). Briefly, the conditions for analyzing anandamide and [$^2H_8$]-anandamide were: atmospheric pressure chemical ionization (APCI) mode, selected-ion-monitoring (SIM) 348.3, 356.3, drying gas 7 l/min, fragmentor 50 V, corona 7 mA. The parameters for analyzing 2-AG in brief were: APCI mode, SIM 379.3, drying gas 7 l/min, fragmentor 70 V, corona 8 mA. Synthetic standards of the three compounds were also analyzed to obtain retention times and recovery efficiency.

2.7. Data analysis

Net agonist-stimulated [^{35}S]GTPγS binding values were calculated by subtracting basal binding values (obtained in the absence of agonist) from agonist-stimulated values (obtained in the presence of agonist) and percent stimulation was determined by dividing net values by the respective basal binding values. Antagonist K_e values were determined by the equation: $K_e = [\text{Ant}]/(\text{CR}-1)$, where [Ant] is the concentration of antagonist and CR is the ratio of the agonist EC_{50} values in the presence and absence of antagonist. The amount of [^{3}H]-D-aspartic acid released from each well at each time point was normalized to the total amount of [^{3}H]-D-aspartic acid incorporated in each well (total released plus the amount remaining after the assay). Basal [^{3}H]-D-aspartic acid release was determined by taking the mean of the last three time points before KCl-induced release began (12–18 min) for each condition (a total of 9 values per condition). Net high [KCl]-induced release was determined by subtracting the basal release value from the amount released in the presence of high [KCl] at each time point. Percent of control values were calculated by taking the mean of the five time points which showed the greatest amount of release in the no drug (control) condition (typically 24–36 min) for each condition (a total of 15 values per condition), dividing the value obtained in the presence of each drug by the value obtained in the control condition and multiplying by 100%.

Specific binding of [^{35}S]GTPγS to intact cells was determined by subtracting the amount of binding obtained in the presence of 25 μM unlabeled GTPγS (non-specific) from values obtained in its absence. Basal binding values were defined as that occurring in the absence of drugs. Drug effects were determined as net agonist-stimulated binding by subtracting basal binding values or were normalized to the amount of basal binding and expressed as percent of basal. Anandamide and 2-AG contents were determined by normalizing to the percentage of [$^2H_8$]-anandamide standard recovered to obtain femtomoles of endocannabinoid and to the milligram of protein in each well from which the effluent or cell fractions were extracted, yielding fmol endocannabinoid/mg cell protein. Significant drug effects were determined by ANOVA (in the case of multiple conditions), Student's *t*-test versus control or a one-sample *t*-test versus a hypothetical value (e.g. percent stimulation by agonist versus 0% stimulation) at $p < 0.05$. Unless otherwise indicated, all data presented are mean ± SEM of at least three experiments performed in duplicate or triplicate.

3. Results

3.1. [^{35}S]GTPγS binding to intact cells and cell membrane homogenates

Rat CGCs have been used as a model primary culture system for the study of neuronal effects of cannabinoids. Previous studies (Pacheco et al., 1993) have shown that cannabinoid agonists inhibit adenylyl cyclase in CGCs, but little is known of the effects of cannabinoids on [^{35}S]GTPγS binding in these cells, a measurement that is more sensitive to differences in ligand efficacy. In order to replicate more closely the conditions of the release assays described below and to

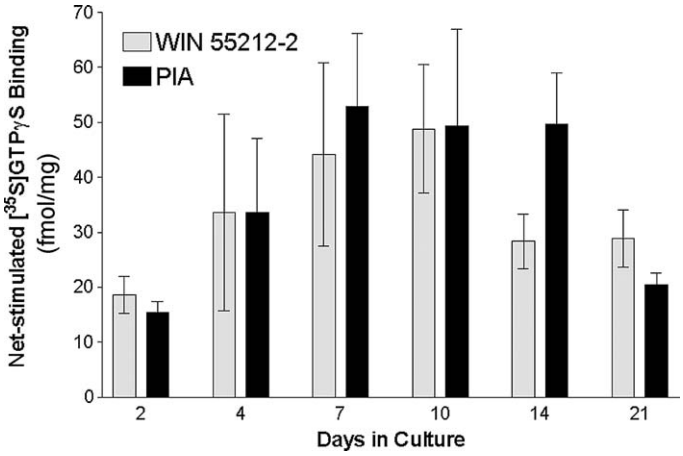

Fig. 1. Time-course of the stimulation of [^{35}S]GTPγS binding to saponin-permeabilized cerebellar granule cells. Cells were first incubated with saponin then with [^{35}S]GTPγS, GDP and 10 μM WIN55212-2 or 1 μM phenylisopropyladenosine to determine the maximal activation of G-proteins by cannabinoid or adenosine receptors, after the cells have been in culture for 2, 4, 7, 10, 14, or 21 days. Data are mean ± SEM from three to five experiments that were each performed in triplicate.

find evidence of the effects of cannabinoids produced endogenously by these cells, [^{35}S]GTPγS binding was performed on CGCs that were essentially intact in culture dishes using a novel modification of this technique. Since [^{35}S]GTPγS does not readily cross a cell membrane to reach the intracellular G-proteins, the cells were permeabilized by a brief (2 min) incubation with saponin. Following this incubation, the remaining assay components were added. Assays were also performed in membrane homogenates from cultured CGCs for comparison under conditions that should remove most of the endogenously produced cannabinoid ligands.

Preliminary assay conditions for saponin treatment were determined (data not shown) using CHO cells transfected with mu opioid receptors (Selley et al., 1997). These studies demonstrated that incubation with 0.1 mg/ml saponin for various times increased specific binding of [^{35}S]GTPγS. An incubation time of 2 min produced an optimal stimulation of binding by agonist of 121% above basal, compared to no specific stimulation observed without saponin treatment. Increased specific binding and agonist stimulation was observed despite the decreased amounts of protein recovered from the saponin-treated wells (~80% decrease). Experiments using the same conditions in CGCs confirmed that saponin pre-treatment dramatically increased specific binding of [^{35}S]GTPγS and agonist stimulation of this binding.

The development of cannabinoid receptor activity in CGCs was determined as the cells differentiated and formed synapses with one another in vitro. Cells were assayed for agonist-stimulated [^{35}S]GTPγS binding to intact CGCs after 2, 4, 7, 10, 14 or 21 days in culture. These experiments revealed that stimulation by either WIN55212-2 or the adenosine A_1 receptor agonist phenylisopropyladenosine was maximal at 7 and 10 days (Fig. 1). Thus, all subsequent experiments, including [^{3}H]-D-aspartic acid release and endocannabinoid measurement experiments, were performed with CGCs during the second week in culture (between 7 and 11 days).

In intact cells (permeabilized by saponin), 3 μM and 10 μM WIN55212-2 stimulated [^{35}S]GTPγS binding to saponin-permeabilized CGCs by 73–74%, while 10 μM Δ^9-tetrahydrocannabinol stimulated by 24%. Interestingly, CB_1 antagonists significantly inhibited basal [^{35}S]GTPγS binding, as both 1 μM SR141716A and 1 μM AM281 decreased binding by 22–23% (Table 1).

Table 1
Effects of cannabinoid ligands on the binding of [^{35}S]GTPγS to saponin-permeabilized cultured cerebellar granule cells or cell membrane homogenates

Ligand, concentration	% Basal [^{35}S]GTPγS binding	
	Intact CGCs	CGC membranes
THC, 10 μM	124 ± 1*	ND
WIN55212-2, 10 μM	173 ± 9***	144 ± 1**
SR141716A, 1 μM	77 ± 6*	96 ± 0.8
AM281, 1 μM	78 ± 4**	ND

Cannabinoid agonists and antagonists were assayed for their effect on [^{35}S]GTPγS binding to intact cultured cerebellar granule cells that were pretreated with saponin or membrane homogenates from cerebellar granule cells as described in "Materials and methods". Data are expressed as %basal [^{35}S]GTPγS binding, determined in the absence of drug and defined as 100%. Results from saponin-permeabilized intact cells are mean values ± SEM from three to five separate experiments, each conducted in triplicate. Results from cerebellar granule cell membrane homogenates are from two experiments. ND, not determined; THC, Δ^9-tetrahydrocannabinol.

* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$ by one-sample t-test versus 100%.

To determine whether the presence of endocannabinoids in these permeabilized cells affected [^{35}S]GTPγS binding, the effects of pre-treatment with the amidase inhibitor PMSF were also assessed in this assay. Data (not shown) revealed that PMSF produced a slight, but not significant, increase in [^{35}S]GTPγS binding. Basal [^{35}S]GTPγS binding was 129 ± 22 and 156 ± 11 fmol/mg in the absence and presence of PMSF, respectively. Similarly, [^{35}S]GTPγS binding with 1 μM AM281 was 102 ± 14 and 123 ± 8 fmol/mg in the absence and presence of PMSF.

In addition to conducting the experiments in intact CGC cells, which have intact biosynthetic ability for endocannabinoids, assays were also conducted in CGC cell membrane homogenates under conditions that should leave little or no endocannabinoid and should disrupt their synthetic capacity. In membrane homogenates from cultured CGCs, 10 μM WIN55212-2 stimulated [^{35}S]GTPγS binding by 44% over basal, while 1 μM SR141716A had no significant effect on basal [^{35}S]GTPγS binding (Table 1). The results for the antagonists were comparable to those obtained in membranes prepared from adult rat cerebellum, in which neither SR141716A (Breivogel et al., 1998) nor AM251 (-5.1 ± 2.8%, current study) up to 1 μM concentrations had any effect on [^{35}S]GTPγS binding although 10 μM WIN55212-2 stimulated binding by 222 ± 18% (data not shown, current study).

3.2. Agonist effects on [^{3}H]-D-aspartic acid release from CGCs

The use of a multi-well superfusion apparatus permitted the simultaneous monitoring of [^{3}H]-D-aspartic acid release from 12 wells of live cultured neurons, providing data on up to four different conditions that were each performed in triplicate. We obtained data for release under both isotonic and high-[KCl]-induced depolarization conditions. While the drugs used in this study did not show any significant effect on basal (isotonic) release of [^{3}H]-D-aspartic acid, significant effects of CB_1 receptor agonists, indirect agonists and antagonists on high-[KCl]-induced release were measured. As a control, the solvents used to dissolve these cannabinoid ligands, ethanol and DMSO were assayed at the maximum concentrations present in the assays with the ligands, and were found to have no affect on basal or high-[KCl]-induced [^{3}H]-D-aspartic acid release (data not shown). Fig. 2 depicts the data from a typical experiment, showing mean data ± SEM for the triplicates of the time-course of release in the presence of two cannabinoid agonists, Δ^9-tetrahydrocannabinol and WIN55212-2 (10 μM each). The high [KCl] Krebs–Ringer buffer was introduced at 12 min, but took at least 6 min to reach the wells through the tubing, such that the increase in release was not observed until the

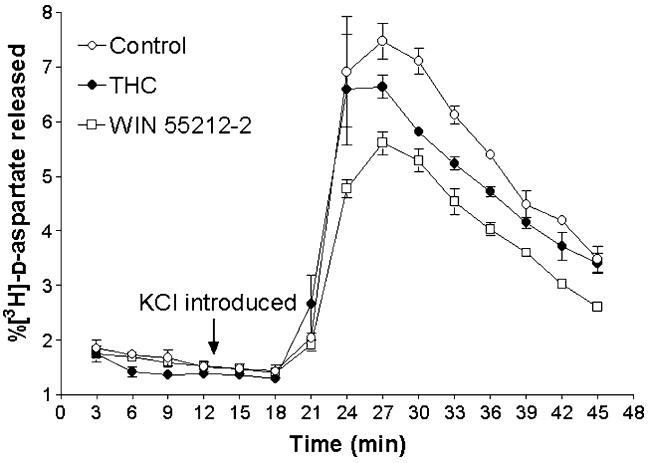

Fig. 2. Results of a typical assay of cannabinoid effects on [^{3}H]-D-aspartate release from cultured cerebellar granule cells. Effects of 10 μM WIN55212-2 and 10 μM Δ^9-tetrahydrocannabinol (THC) are shown in comparison to basal (no drug). For each well, the total amount of [^{3}H]-D-aspartate taken up by the cells was determined by adding the total amount released to the amount remaining in the cells after the assay was completed. Data are mean ± SEM from triplicate determinations of each condition of the amount of [^{3}H]-D-aspartate released during each 3 min time period normalized to the total amount of [^{3}H]-D-aspartate taken up by the cells in each well.

21 min time point. Release typically peaked between 24 and 36 min, and then declined over the course of the collection (Fig. 2).

Inhibition of the release of [^{3}H]-D-aspartic acid by WIN55212-2 was concentration-dependent and the maximal effect of WIN55212-2 was greater than that of Δ^9-tetrahydrocannabinol (Table 2). WIN55212-2 failed to inhibit [^{3}H]-D-aspartic acid release at 0.1 μM but inhibited release by 13% at 1 μM and by 33% at

Table 2
Effect of cannabinoid agonists and indirect agonists on KCl-induced [^{3}H]-D-aspartate release from cultured cerebellar granule cells

Drug, concentration	% Control release
WIN55212-2, 0.1μM	101 ± 0.9
WIN55212-2, 1.0 μM	87 ± 11
WIN55212-2, 10 μM	$67 \pm 3.4^{**}$
THC, 10 μM	$84 \pm 5.7^{*}$
PMSF, 50 μM (pre-treatment)	73 ± 7.7
AM404, 1 μM	$75 \pm 5.0^{*}$

Different concentrations of WIN55212-2 were compared to a maximally effective concentration of Δ^9-tetrahydrocannabinol (THC) for the inhibition of [^{3}H]-D-aspartate release from cultured cerebellar granule cells. The effects of a pre-treatment with the amidase inhibitor phenylmethylsulfonyl fluoride (PMSF) and the putative endocannabinoid transport inhibitor AM404 were also assessed. Data were normalized to the amount of KCl-induced release obtained under control conditions (defined as 100%) and expressed as percent of control ± SEM. Results are mean values ± SEM from three to 21 separate experiments, each conducted in triplicate.

* $p < 0.05$.

** $p < 0.01$ versus control.

10 μM, while Δ^9-tetrahydrocannabinol inhibited release by 16% at 10 μM. It appears that WIN55212-2 was approximately twice as efficacious as Δ^9-tetrahydrocannabinol ($p < 0.05$, Student's t-test of 10 μM WIN55212-2 versus Δ^9-tetrahydrocannabinol), because Δ^9-tetrahydrocannabinol and WIN 5512-2 have each been shown to be maximally effective for the stimulation of [^{35}S]GTPγS binding and inhibition of adenylyl cyclase at 10 μM (Breivogel and Childers, 2000; Breivogel et al., 1998).

To confirm that the inhibition of [^{3}H]-D-aspartic acid release by cannabinoid ligands was mediated by cannabinoid receptors, 1 μM of the CB_1-selective antagonist SR141716A was combined with 10 μM WIN55212-2. The antagonist not only blocked the inhibition of release by the agonist, which was inhibited by 32 ± 2% in these experiments, but also produced release in excess of that observed under control conditions (Fig. 3), for a net increase of 32 ± 15% in [^{3}H]-D-aspartic acid release in the presence of WIN55212-2 plus SR141716A. In subsequent experiments conducted in the absence of an agonist, SR141716A stimulated [^{3}H]-D-aspartic acid release (Fig. 3). in a concentration-dependent manner, occurring at concentrations as low as 0.1 nM (Table 3).

In order to determine whether this increase in [^{3}H]-D-aspartic acid release was specific to SR141716A, which has previously been reported to exhibit inverse agonist activity in other types of assays (Bouaboula et al., 1997; Landsman et al., 1997), an additional cannabinoid receptor antagonist, AM281, was assayed. To obtain an estimate of this ligand's functional affinity at cerebellar cannabinoid receptors, it was first assayed for the ability to shift the concentration–effect curve of

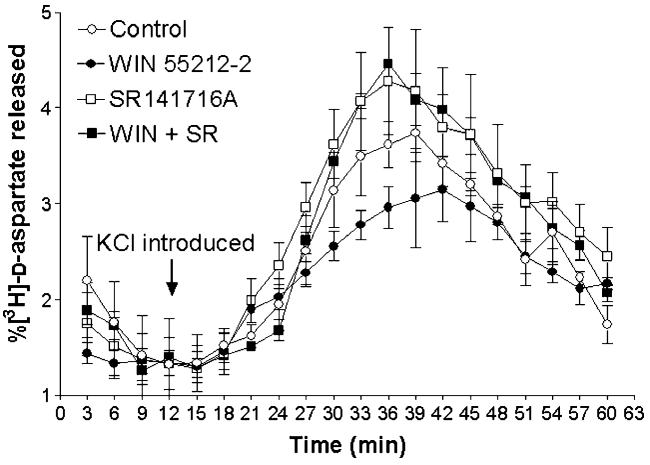

Fig. 3. Results of a typical assay of the effect of CB_1 antagonist on [^{3}H]-D-aspartate release from cultured cerebellar granule cells. Effect of 10 μM WIN55212-2, 10 nM SR141716A and 10 μM WIN55212-2 + 1 μM SR141716A are shown in comparison to control (no drug). Data were analyzed and normalized as in Fig. 2.

Table 3
Effect of cannabinoid antagonists on KCl-induced [^{3}H]-D-aspartate release from cultured cerebellar granule cells

Antagonist, concentration	% Control release
SR141716A, 0.1 nM	119 ± 18
SR141716A, 1.0 nM	122 ± 11*
SR141716A, 10 nM	128 ± 6.0**
AM281, 100 nM	121 ± 6.1**
AM630, 10,000 nM (10 μM)	380 ± 84*

Different concentrations of SR141716A and single concentrations of AM281 and AM630 were assayed for the effects on [^{3}H]-D-aspartate release from cultured cerebellar granule cells. Data were normalized to the amount of KCl-induced release obtained under control conditions (defined as 100%) and expressed as percent of control ± SEM. Results are mean values ± SEM from three to 14 separate experiments, each conducted in triplicate.

* $p < 0.05$.

** $p < 0.01$ versus control.

WIN55212-2 for the stimulation of [^{35}S]GTPγS binding to membranes prepared directly from adult rat cerebellum (data not shown). Similar to results obtained with SR141716A in cerebellar membrane homogenates, AM281 alone did not affect [^{35}S]GTPγS binding, and exhibited a K_e value of 0.92 ± 0.72 nM for antagonism of WIN55212-2 activity. Thus, 100 nM AM281 was used to assure near complete occupancy of cannabinoid receptors. AM281 (100 nM) administered alone resulted in a 21% increase in depolarization-evoked [^{3}H]-D-aspartic acid release (Table 3), similar to the increases observed with SR141716A.

The cannabinoid CB_1 receptor antagonists increased the release of [^{3}H]-D-aspartic acid, an effect opposite to that produced by WIN55212-2 or Δ^9-tetrahydrocannabinol, both of which decreased release. Hence, the antagonists were either blocking the effects of endogenous cannabinoids produced by the CGCs, or acting as inverse agonists. To determine which of these effects was responsible for the actions of the antagonists, further experiments were conducted using two indirect agonists: the irreversible amidase inhibitor PMSF and the putative anandamide transport inhibitor AM404 (Table 2 and Fig. 4). Exposing the cells to 1 μM AM404 decreased [^{3}H]-D-aspartic acid release to 75 ± 5% of control ($n = 3$, $p < 0.05$). Pre-treatment of the cultured cells with PMSF resulted in a similar trend—decreasing [^{3}H]-D-aspartic acid release to 73 ± 8% ($n = 3$, $p = 0.0713$) of that seen in control cells.

3.3. Determination of endogenous cannabinoids in CGCs by lipid extractions

In both the [^{3}H]-D-aspartic acid release and [^{35}S]GTPγS binding experiments, cannabinoid antagonists alone produced effects that were opposite to those of agonists. To directly test the hypothesis that the

antagonists were blocking the effects of endogenous cannabinoid agonists present in these cell preparations, lipid extracts of cultured CGCs and the buffer in which they were incubated were prepared and analyzed for anandamide and 2-AG content by LC–MS. To facilitate data interpretation, the cells were prepared in the same way and exposed to the same buffers (either a non-depolarizing or depolarizing concentration of KCl) as those used in the assays of [^{3}H]-D-aspartic acid release. Since PMSF was implicated to increase endocannabinoid levels in [^{3}H]-D-aspartate release and intact cell [^{35}S]GTPγS binding assays, the effects of PMSF pre-treatment on cellular anandamide and 2-AG content were also determined. LC/MS analysis revealed the presence of anandamide and 2-AG under basal conditions in both cells and buffer, with higher levels of 2-AG compared to anandamide. Pre-treatment with PMSF increased the amount of anandamide and 2-AG extracted from the cells by 124 ± 13 and $306 \pm 110\%$, respectively ($p < 0.05$ each). The treatment also increased the amount of anandamide in the effluent by $197 \pm 37\%$ ($p < 0.05$), but had no apparent effect on the amount of 2-AG recovered from the effluent ($-6 \pm 28\%$).

Since PMSF treatment increased the amounts of anandamide and 2-AG, experiments to determine the effects of depolarization were conducted in PMSF-pretreated cells. In preparations not exposed to the depolarizing stimulus, on average 81.0 fmol/mg of anandamide and 83,000 fmol/mg of 2-AG were found in the cells, while 129 fmol/mg of anandamide and 3040 fmol/mg of 2-AG were found in the buffer. In preparations exposed for 1 h to a depolarizing concentration of KCl (100 mM), anandamide content of the cells was lower by 25%, and 2-AG content of the cells was lower by 94% ($p < 0.05$). In contrast, the amounts of anandamide and 2-AG in the buffer were not significantly affected by depolarization of the cells (Table 4).

Table 4
Endogenous cannabinoid ligands in cerebellar granule cell cultures

Sample	Non-depolarized	Depolarized
Anandamide in cells (fmol/mg)	81.0 ± 4.8	$60.5 \pm 3.0^*$
Anandamide in buffer (fmol/mg)	129 ± 9	119 ± 9
2-AG in cells (fmol/mg)	$83,000 \pm 26,100$	$4870 \pm 560^*$
2-AG in buffer (fmol/mg)	3040 ± 440	3090 ± 190

Lipid extracts of cultured cerebellar granule cells and the incubation buffers were analyzed for endogenous cannabinoid ligand content by LC/MS as described in "Materials and methods". Extracts were prepared after a 1 h incubation of the cells in isotonic Krebs–Ringers buffer containing either a non-depolarizing (5 mM) or depolarizing (100 mM) concentration of KCl. Each treatment and extraction was performed in quadruplicate. 2-AG, 2-arachidonoyl glycerol.

* $p < 0.05$ versus non-depolarizing conditions.

4. Discussion

It has been shown that cannabinoids activate G-proteins and inhibit the release of a number of transmitters in the central and peripheral nervous systems via the activation of presynaptic cannabinoid receptors including CB_1 (Schlicker and Kathmann, 2001), and potentially at least one other receptor that is sensitive to anandamide and WIN55212-2 (Breivogel et al., 2001; Hajos and Freund, 2002; Hajos et al., 2001). Electrophysiological effects of cannabinoids on postsynaptic neurons in cerebellar slices were consistent with a decrease in presynaptic neurotransmitter release (Levenes, 1998; Takahashi and Linden, 2000). The present study is the first to demonstrate a modification of the agonist-stimulated [^{35}S]GTPγS binding assay to measure activation of G-proteins in cultured cells that are essentially intact, and also to directly measure the amount of neurotransmitter released as it is affected by cannabinoid, as previously examined in other cultured neurons (for review see, Schlicker and Kathmann, 2001).

WIN55212-2 and Δ^9-tetrahydrocannabinol displayed significant activity in the intact cell [^{35}S]GTPγS binding assays, stimulating binding by 73 and 24% over basal binding values, respectively. WIN55212-2 and Δ^9-tetrahydrocannabinol also inhibited the depolarization-evoked release of [^{3}H]-D-aspartate from CGCs by up to 33 and 16%, respectively. The maximally effective concentration of WIN55212-2 (10 μM) was identical to that shown to be maximally effective for the activation of G-proteins, displacement of [^{3}H]cannabinoid ligand binding, or inhibition of adenylyl cyclase activity in brain membranes (Breivogel and Childers, 2000; Breivogel et al., 1997b). Similarly, 10 μM Δ^9-tetrahydrocannabinol has been shown to be a maximally effective concentration for the activation of G-proteins or inhibition of adenylyl cyclase (Breivogel and Childers, 2000). The lower efficacy of Δ^9-tetrahydrocannabinol compared to WIN55212-2 observed in this study agrees with previous results of G-protein activity and adenylyl cyclase inhibition where Δ^9-tetrahydrocannabinol was a partial agonist compared to other cannabinoid receptor agonists (Breivogel and Childers, 2000; Breivogel et al., 1998; Burkey et al., 1997; Sim et al., 1996a).

While [^{35}S]GTPγS binding measures the first step in the signal transduction process, an increase in apparent receptor reserve and additional signal amplification at subsequent steps in the pathway, such as alterations in adenylyl cyclase, mitogen-activated protein kinase (MAPK) or ion channel activity, can diminish or eliminate differences in apparent efficacies among agonists. At each step it is possible that a given signal transduction component becomes maximally activated (or closer to maximally activated) by agonists that were

only partial agonists for stabilizing the active conformation of the receptor. This principle was shown to apply to Δ^9-tetrahydrocannabinol and WIN55212-2 in cerebellar membranes, since Δ^9-tetrahydrocannabinol produces 20% of the effect of WIN55212-2 in the agonist-stimulated [^{35}S]GTPγS binding assay, but 50% of the effect of WIN55212-2 on the inhibition of adenylyl cyclase activity (Breivogel and Childers, 2000). This could explain results in the present study as well, since the effect of Δ^9-tetrahydrocannabinol on [^{35}S]GTPγS binding to intact cells was 32% of the effect of WIN55212-2, while the effect of Δ^9-tetrahydrocannabinol on neurotransmitter release from these cells was 48% of the effect of WIN55212-2.

The selective CB_1 antagonists SR141716A and AM281 exhibited activity that opposed that observed with agonists by decreasing basal [^{35}S]GTPγS binding to intact cells by 22–23%, and not only reversed the inhibition of [^{3}H]-D-aspartate release by WIN 55,212-2, but increased the amount of depolarization-invoked [^{3}H]-D-aspartate released over basal levels by 21–28%. These findings can be interpreted as antagonism of spontaneous receptor activity (i.e. inverse agonism) or blockade of activity of endogenous ligands at cannabinoid receptors. Although there have been reports of inverse agonist activity of SR141716A in other systems, in this study the effects of the amidase inhibitor PMSF and the anandamide transport inhibitor AM404 on [^{3}H]-D-aspartate release (decreased by 25%) and the detection of endocannabinoids (increased two- to fourfold), support the hypothesis that the effects seen with the antagonists were due to blockade of endocannabinoid activity. The observation that PMSF pre-treatment and AM404 exposure each increased endocannabinoid (anandamide and 2-AG) levels and decreased depolarization-induced [^{3}H]-D-aspartate release, implies that increased endocannabinoid levels were responsible for the observed effects on [^{3}H]-D-aspartate release. While the effect of PMSF can more confidently be attributed to inhibition of endocannabinoid degradation by amidases (e.g. FAAH), significant doubt has recently been cast on the existence of an endocannabinoid transporter and AM404 has been shown to be a competitive inhibitor of FAAH (Glaser et al., 2003), implying that the increased action of endocannabinoids observed with AM404 has more likely been due to inhibition of degradation (Fig. 4). It is certainly a possibility in the current study that PMSF and AM404 worked by similar mechanisms, since the magnitude of the effects of PMSF and AM404 on [^{3}H]-D-aspartate release were nearly identical. Blockade of endocannabinoid tone by SR141716A and AM281 in intact CGCs is also supported by the observation that these antagonists alone did not affect [^{35}S]GTPγS binding to brain membrane homogenates, preparations from which the endogenous ligands have

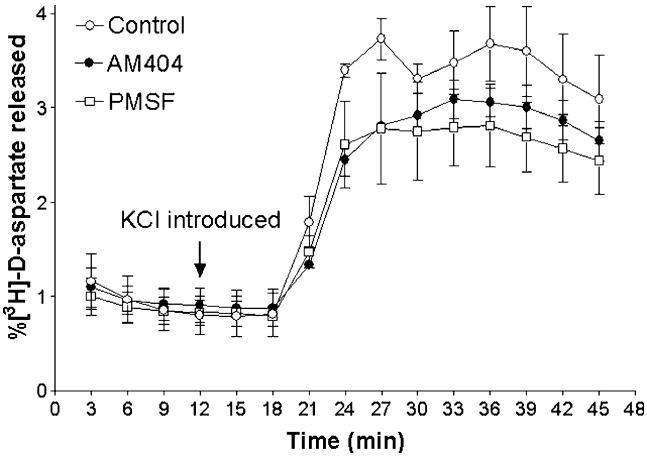

Fig. 4. Results of a typical assay of the effects of the pre-treatment with the irreversible fatty acid amide hydrolase (FAAH) inhibitor phenylmethylsulfonyl fluoride (PMSF) or exposure to the endocannabinoid transport inhibitor (and competitive FAAH inhibitor) AM404 on [^{3}H]-D-aspartate release from cultured cerebellar granule cells. PMSF was added to the appropriate wells at 50 μM during the 15 min incubation with [^{3}H]-D-aspartate; cells were subsequently rinsed before addition of ligands and initiation of perfusion. Effect of PMSF pre-treatment and 1 μM AM404 are shown in comparison to control (no drug). Data were analyzed and normalized as in Fig. 2.

presumably been removed by centrifugation and disposal of supernatants. PMSF pre-treatment did not significantly affect basal [^{35}S]GTPγS binding or the effect of AM281 on [^{35}S]GTPγS binding to intact CGCs, but there was a trend towards an increase, implying some effect of endocannabinoids in that assay as well.

Several recent studies have indicated that endogenous cannabinoids act as retrograde neuromodulators; i.e. neuronal excitation stimulates their release from the postsynaptic neuron from which they travel back to the presynaptic neuron, suppressing further release of neurotransmitter. This has been implicated as a mechanism for depolarization-induced suppression of inhibition (or long-term depression) in cerebellar Purkinje cells (Kreitzer and Regehr, 2001a) and in hippocampus (Wilson and Nicoll, 2001). However, these cultures are nearly pure CGCs (primary glutamaturgic inputs to the Purkinje cells), which form synapses with each other in culture. Our data indicated that the cellular content of both anandamide and 2-AG was significantly decreased by depolarization, implying endocannabinoid release from the neurons. Whether the endocannabinoids inhibited presynaptic neurons by traveling backwards across the synapse or acted upon the neurons producing the endocannabinoids could not be determined. The lack of detectable changes in the incubation medium could indicate that they were degraded by metabolizing enzymes before the 1 h time point or could be attributed to the high lipophilicity of these compounds. High lipophilicity prohibits the accumulation of appreciable concentrations of endocannabinoid in

aqueous solution before redistributing to other surfaces such as the walls of the culture dish. The fact that PMSF pre-treatment increased 2-AG content in the lipophilic (cellular) phase, but not the aqueous (effluent) phase, supports the notion that the 2-AG content in the aqueous phase was near saturation. Anandamide levels were increased in the cells and the effluent by PMSF, but the levels of anandamide were much lower than the levels of 2-AG in either phase, making it more feasible to observe an increase in anandamide concentration in the aqueous phase. No further increase in anandamide was observed by depolarization (which was performed following PMSF pre-treatment) perhaps due to the anandamide levels already being near saturation. In any case, the release of both anandamide and 2-AG in the cells upon depolarization indicates a high likelihood of heightened synaptic activity of endocannabinoids. The greater magnitude of change in 2-AG levels compared to that of anandamide levels suggests a greater role of 2-AG in endocannabinoid signaling in this system.

In summary, this study characterized aspects of cannabinoid signaling in primary cultures of CGCs. Using an intact-cell version of the agonist-stimulated [^{35}S]GTPγS binding assay, it was shown that cannabinoid agonists stimulate and antagonists inhibit the activation of G-protein alpha subunits in these cells. Cannabinoid agonists and indirect agonists (FAAH inhibitors and/or uptake inhibitor) inhibited the release of glutamate from cerebellar granule neurons, while antagonists increased basal release using direct measurement of the efflux of an analog of the neurotransmitter. Finally, measurement of the levels of endocannabinoids, anandamide and 2-AG, the effect of depolarization, and the effects of cannabinoid antagonists on glutamate release, all support the notion that endocannabinoids (anandamide and 2-AG) are active in CGCs, and that neural stimulation further activates the endocannabinoid system to suppress glutamate release, possibly via a retrograde mechanism.

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