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Cultured Rat Microglial Cells Synthesize the Endocannabinoid 2-Arachidonylglycerol, Which Increases Proliferation via a CB_2 Receptor-Dependent Mechanism

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ABSTRACT

Microglia, as phagocytes and antigen-presenting cells in the central nervous system, are activated in such disease processes as stroke and multiple sclerosis. Because peripheral macrophages are capable of producing endocannabinoids, we have examined endocannabinoid production in a macrophage-colony stimulating factor (M-CSF)–dependent rat microglial cell line (RTMGL1) using reversed phase high-pressure liquid chromatography and liquid chromatography-mass spectroscopy. We determined that cultured microglial cells produce the endocannabinoid 2-arachidonylglycerol (2-AG) as well as anandamide in smaller quantities. When 2-AG, but not anandamide, is added exogenously, RTMGL1 microglia increase their proliferation. This increased proliferation is blocked by an antagonist of the CB_2 receptor *N*-[(1*S*)-endo-1,3,3-trimethyl bicyclo heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide (SR144528) and mimicked by the CB_2 receptor-specific agonist 1,1-dimethylbutyl-1-deoxy-Δ^9-tetrahydrocannabinol (JWH133). Accompanying the increase in proliferation seen with 2-AG is an increase in active ERK1 that is also blocked with SR144528. The RTMGL1 microglial cells, which exist in a primed state, express the CB_1 and CB_2 receptors as demonstrated by reverse transcription-polymerase chain reaction and immunostaining. The CB_2 receptor in untreated cells is expressed both at the cell surface and internally, and exposure of the cells to 2-AG significantly increases receptor internalization. These data suggest that 2-AG activation of CB_2 receptors may contribute to the proliferative response of microglial cells, as occurs in neurodegenerative disorders.

Microglia, as the primary immune effector cells in the central nervous system, proliferate and migrate to sites of infection, inflammation, or tissue injury in response to astrocyte secretion of macrophage colony-stimulating factor (M-CSF) (Sawada et al., 1990; Shafit-Zagardo et al., 1993; Kloss et al., 1997). When fully activated, microglia produce nitric oxide, tumor necrosis factor-α, and other cytokines and become phagocytic (Streit et al., 1999). Initially, microglia are thought to play a protective role, clearing debris and allowing regeneration of viable axons after brief episodes of neuronal injury, as occurs immediately after physical trauma or stroke. However, it is becoming apparent that sustained activation of microglia contributes to the pathogenesis of chronic neurodegenerative diseases such as Alzheimer's and Parkinson's diseases (Streit et al., 1999). Because increased numbers of microglia are seen in brains of patients with multiple sclerosis (Schonrock et al., 1998), Alzheimer's disease (Mackenzie et al., 1995), and HIV (Gendelman et al., 1994), and because sustained microglial activation as seen in inflammatory diseases of the central nervous system is known to have deleterious effects on the surrounding neurons (Nelson et al., 2002), factors mediating microglial proliferation and activation are of intense interest.

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ABBREVIATIONS: M-CSF, macrophage-colony stimulating factor; Δ^9-THC, Δ^9-tetrahydrocannabinol; AEA, *N*-arachidonylethanolamine; 2-AG, 2-arachidonylglycerol; SR141716, *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboximide hydrochloride; SR144528, *N*-[(1*S*)-endo-1,3,3-trimethyl bicyclo heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide; JWH133, 1,1-dimethylbutyl-1-deoxy-Δ^9-tetrahydrocannabinol; DMEM, Dulbecco's modified Eagle's medium; HPLC, high-pressure liquid chromatography; LC-MS, liquid chromatography-mass spectroscopy; FACS, fluorescence-activated cell sorting; A23187, 4-bromocalcimycin; 1-AG, 1(3)-arachidonylglycerol; PBS, phosphate-buffered saline; TCA, trichloroacetic acid; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; TBS, Tris-buffered saline; ERK, extracellular signal-regulated kinase; PBST, phosphate-buffered saline/Tween 20; DAG, diacylglycerol; RHC 80267, 1,6-*bis*(cyclohexyloximinocarbonylamino)hexane; GPCR, G-protein coupled receptor; DAPI, 4,6-diamidino-2-phenylindole; PD98059, 2′-amino-3′-methoxyflavone; ANOVA, analysis of variance.

The immunomodulatory effects of cannabinoids have been well studied in recent years; in general, marijuana and its active constituent Δ^9-tetrahydrocannabinol (Δ^9-THC) have been shown to impair the cell-mediated immune response (for review, see Berdyshev, 2000). Many of these effects are mediated by the CB_2 receptor, as shown by the finding that Δ^9-THC inhibits helper T-cell activation by normal, but not CB_2 receptor knockout-derived, macrophages (Buckley et al., 2000). However, most studies examining effects of cannabinoids on CB_2 receptor-expressing immune cells have focused on Δ^9-THC; to date, less is known about the effects of endogenously produced cannabinoids (endocannabinoids) on immune cells.

Endocannabinoids, including *N*-arachidonylethanolamine (anandamide, AEA) and 2-arachidonylglycerol (2-AG), are produced in an autocrine or a paracrine manner by both immune cells and cells of the nervous system (reviewed in Fride, 2002; Salzet et al., 2000). Both AEA and 2-AG bind with moderate affinity to the G-protein-coupled CB_1 (found in the central nervous system) and CB_2 (found in the periphery) receptors and exert most of their known effects via the pertussis toxin-sensitive $G\alpha_{i/o}$ proteins. Although AEA does bind to both cannabinoid receptors, it has very low efficacy at the CB_2 receptor (Hillard et al., 1999). Recently, it has been demonstrated that mouse microglia produce a number of putative endocannabinoids (Walter et al., 2003). Given the immunomodulatory role of cannabinoids, we sought to better understand how the endocannabinoids AEA and 2-AG regulate microglial function.

In the normal brain, microglia exist primarily in a quiescent, resting state; however, when dissociated from the brain and cultured in vitro, microglia heterogeneously gain an activated phenotype (Slepko and Levi, 1996). To counteract these issues and obtain consistent data, we have developed a M-CSF-dependent, nontransformed rat microglial cell line (RTMGL1) that exists in a primed, proliferative state. Here, we demonstrate that RTMGL1 microglial cells produce the endocannabinoid 2-AG and, like other primed microglia (Carlisle et al., 2002), express the CB_2 receptor at easily detectable amounts and the CB_1 receptor in lower amounts. Furthermore, we have found that exogenous 2-AG, but not AEA, increases proliferation of the M-CSF–dependent RTMGL1 microglial cell line and that this effect is mediated by the CB_2 receptor.

Materials and Methods

Reagents. The endocannabinoids 2-AG and AEA were purchased from Research Biochemicals International (Natick, MA) and Cayman Chemical (Ann Arbor, MI), respectively. SR141716 and SR144528 were a generous gift from Sanofi Recherche (Montpellier, France). JWH133 was the kind gift of Dr. John Huffman (Clemson, SC). Media for cell culture were purchased from Invitrogen (Carlsbad, CA); culture flasks and plates were purchased from Corning Life Sciences (Acton, MA). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated.

M-CSF Source. Conditioned medium from the transformed bone marrow-derived LADMAC cell line (cells provided by Dr. William Walker of St. Jude Children's Research Hospital, Memphis, TN) was used as an M-CSF source for microglial cultures, as described by Sklar et al. (1985). This medium was filtered and titrated for potency in inducing RTMGL1 growth using a [^{3}H]thymidine incorporation assay. A single lot of conditioned medium was used for all of these studies.

Rat Microglial Cell Cultures. The RTMGL1 rat microglial cell line was generated as described previously (Rademacher et al., 2004). Briefly, loosely adhering microglia were isolated from a primary culture of mixed glial cells by shaking. These isolated microglia were maintained in a M-CSF–rich medium until a portion of the microglia began to proliferate and form foci. Clones were isolated from individual colonies by limiting dilution, and the clone RTMGL1 was chosen for further study based on its uniform bipolar morphology. Cells were maintained in uncoated Costar culture flasks in Dulbecco's modified essential medium supplemented with glucose (4.5 g/l), L-glutamine, and pyridoxine (DMEM), as well as 10% (v/v) heat-inactivated, characterized fetal bovine serum (Hyclone, Logan, UT), 20% LADMAC-conditioned medium, and 0.1 g/l gentamicin (Invitrogen), with medium changes every 2 to 3 days. The purity of microglial cultures was established by staining as described for the astrocyte marker glial fibrillary acidic protein to identify astrocytes, and characterization of microglial lectin affinity for *Griffonia simplicifolia* isolectin B4 (isoB4) (Streit and Kreutzberg, 1987). Cells were passaged a maximum of 35 times by mechanical removal from flasks.

Endocannabinoid Production. Endocannabinoid production by RTMGL1 cultures was determined by incubating the cells with [^{14}C]arachidonic acid and resolving the ^{14}C metabolites by high-performance liquid chromatography (HPLC) or liquid chromatography-mass spectrometry (LC-MS). RTMGL1 cultures in 75-cm^2 flasks were washed three times with 10 mM HEPES buffer containing 150 mM NaCl, 2 mM $CaCl_2$, 5 mM KCl, 1 mM $MgCl_2$, and 6 mM glucose, pH 7.4, and then incubated with 10 ml of the same buffer. When indicated, inhibitors were added to the cultures and incubated for 10 min at 37°C, after which 0.5 μM [^{14}C]arachidonic acid (810 mCi/mmol; PerkinElmer Life and Analytical Sciences, Wilmington, DE) and 10^{-5} M unlabeled arachidonic acid in ethanol were added to the flasks and the cells incubated at 37°C for 5 min. The cells were treated with 10 μM A23187, a calcium ionophore, for 10 min at 37°C, after which the RTMGL1 cells were mechanically disrupted. Ethanol and glacial acetic acid were added to the buffer/cell mixture at final concentrations of 18% and 1% (v/v), respectively, and the cell debris was removed by centrifugation. Supernatant was applied to a Bond Elut C18 column (Chrom Tech, Apple Valley, MN), and the [^{14}C]arachidonic acid metabolites were eluted with ethyl acetate, evaporated to dryness under N_2, and stored at −80°C before HPLC or LC-MS analysis.

High-Performance Liquid Chromatography. Extracted [^{14}C]arachidonic acid metabolites were analyzed by reversed-phase HPLC (Rademacher et al., 2004). To separate endocannabinoids from the other major eicosanoids, primarily prostaglandins and unmetabolized arachidonic acid, an HPLC system with a Nucleosil-C_{18} (5 μm, 4.6 × 250 mm) column was used. The products were eluted by a linear gradient of 50% solvent A (0.1% glacial acetic acid in acetonitrile) in water (solvent B) to 100% solvent A over 40 min. Flow rate was 1 ml/min, and absorbance was monitored at 235 nm. Column effluent was collected in 0.2-min fractions, and radioactivity of individual fractions was determined by liquid scintillation spectrometry. The retention times of the radioactive peaks was compared in all cases with known standards separated under identical chromatographic conditions.

Mass Spectrometry. The identity and relative quantity of endocannabinoids was determined using electrospray ionization LC-MS after the addition of deuterated AEA and 2-AG. Fractions of column effluent from the HPLC system described above that corresponded to peaks of interest were collected and extracted into 1:1 cyclohexane/ethyl acetate. The organic layer was dried under N_2 and resuspended in 20 μl of methanol for analysis. Samples (5 μl) were analyzed using electrospray ionization LC-MS (1100 LC/MSD, SL model; Agilent, Palo Alto, CA) and separated on a reversed-phase C_{18} column (Kromasil; 250 × 2 mm) using mobile phase A (deionized water, 1 mM ammonium acetate, and 0.005% glacial acetic acid) and mobile phase B (methanol, 1 mM ammonium acetate, and 0.005% glacial acetic

acid). Samples were eluted at a flow rate of 300 μl/min for 25 min by a linear gradient of 85% solvent A to 100% solvent B, then held at 100% solvent B for 10 min. Drying gas flow was 12 l/min, drying gas temperature was 350°C, nebulizer pressure was 35 psi, capillary and fragmentor voltages were 3000 and 120 V, respectively, and vaporizer temperature was 325°C. Selective ion monitoring in positive mode allowed for the detection of AEA (m/z = 346; retention time = 28.05 min), 2-AG and 1(3)-arachidonylglycerol (1-AG) (m/z = 377; retention times = 35.17 min and 36.63 min, respectively). Limits of detection for endocannabinoids were 10 pg/μl (AEA) and 500 pg/μl (2-AG). Because 2-AG is often seen as a doublet because of isomerization to 1-AG, total yield was calculated by adding the areas of both peaks.

[^{3}H]Thymidine Incorporation. A [^{3}H]thymidine incorporation assay was used to measure RTMGL1 proliferation. Cells were grown to 50 to 70% confluence in 12-well culture plates. Growth medium was removed, cells were washed once in DMEM, and medium was replaced with DMEM for a 24-h starvation period. After 24 h, drugs were added in 0.4 to 0.6 μl dimethyl sulfoxide or ethanol. Vehicle control treatments were done for all trials, and neither ethanol nor dimethyl sulfoxide had any demonstrable effect on proliferation at concentrations up to 2 μl/ml. Ten minutes after adding drugs, 2.5% LADMAC conditioned medium was added to cells to stimulate growth. During the last 4 h of treatment, 0.5 μCi/well [^{3}H]thymidine was added to wells in 50 μl of DMEM. After 24 h of growth stimulation, medium was removed from cells and they were washed twice with ice-cold phosphate-buffered saline (PBS, pH 7.4). To assay for incorporated thymidine, cellular macromolecules were precipitated by a 10-min incubation on ice with chilled 10% trichloroacetic acid (TCA). This was removed, and followed by two 5-min incubations with 10% TCA. All TCA was then aspirated, cells were removed from ice, and precipitates were solubilized by adding 0.5 ml of a solution containing 0.2 M sodium hydroxide and 1% (w/v) SDS. Plates were shaken at room temperature for 5 min, after which the solubilized contents of wells were removed into scintillation vials and radioactivity was determined. Each drug treatment in an experiment was expressed as a percentage of control [^{3}H]thymidine incorporation.

RT-PCR. To identify transcript for the CB_1 and CB_2 receptor, poly-A^+ mRNA was isolated from cultured microglia by affinity chromatography according to manufacturer's protocol (QuickPrep Micro mRNA purification kit; Amersham Biosciences, Piscataway, NJ). One microgram of isolated mRNA was reverse-transcribed with 200 units of Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) for 1 h at 37°C in the manufacturer's buffer, containing 0.5 μg of poly(dT) primer and 0.67 mM dNTP. The resulting cDNA was used as a template for PCR reactions. Primers for the CB_1 receptor were designed based on the published sequence of rat CB_1 receptor (GenBank accession no. NM012784.1). The following oligonucleotides were used for PCR amplification of full-length CB_1 receptor coding sequence: 5′-ATGAAGTCGATCCTAGATGGCCTTGCAGA (forward, bases 153–181), and 5′-TCACAGAGCCTCGGCGGACGTG (reverse, bases 1553–1574). For internal amplification of CB_1 receptor sequence, the primers 5′-GATGCAGGCCTTCCTACCACT (forward, bases 598–618) and 5′-CACCACCAGGATCAGAACCAG (reverse, bases 1188–1208) were used. The following primers were used to amplify the CB_2 receptor transcript: 5′-GACCTTCACAGCCTCTGTGGGCA (forward, bases 493–515 of rat sequence with a G-to-A substitution at base 502), and 5′-GGTTTTCACATCAGCCTCTGTTT (reverse, bases 1166–1186 with a C-to-A substitution at base 1177). Primers to glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 5′-CACGGCAAGTTCAATGGCACA (forward) and 5′-GAATTGTGAGGGAGAGTGCTC (reverse) were used in PCR as a positive control for cDNA integrity. All primers were purchased from QIAGEN/Operon Technologies (Alameda, CA).

PCR reactions were carried out in the manufacturer's buffer supplemented with 1.5 mM $MgCl_2$, 0.4 mM dNTP, 1 μM concentrations of forward and reverse primers, and 2.5 U of *Taq* DNA polymerase (Promega). CB_1 receptor transcript was amplified by 40 cycles of denaturation at 95°C (45 s), annealing at 60°C (45 s), and extension at 60°C (60 s). The CB_2 receptor transcript was amplified by 35 cycles of denaturation at 92°C (45 s), annealing at 56°C (45 s), and extension for at 72°C (60 s). The amplification of GADPH was carried out in parallel under both conditions. As a control for DNA contamination of the mRNA isolate, PCR reactions using a template of cDNA mixture lacking Moloney murine leukemia virus reverse transcriptase were amplified under both conditions. All PCR reactions were electrophoresed in 40 mM Tris acetate/ 2 mM EDTA on a 2% agarose (Invitrogen) gel that was then incubated in a 0.5 μg/ml ethidium bromide solution and visualized on a UV transilluminator. Digital images were captured with a Foto/Analyst Archiver electronic documentation system (Fotodyne Inc., Hartland, WI).

Immunocytochemistry. Cells were grown to 50 to 75% confluence on uncoated coverslips (Fisher Scientific, Pittsburgh, PA) placed in 24-well plates, washed in PBS, and fixed with 4% (w/v) paraformaldehyde in PBS. Coverslips were washed in Tris-buffered saline (TBS, pH 7.4) containing 0.1% (w/v) Triton X-100 and incubated in a solution of TBS-Triton with 4% (v/v) normal goat serum (TBS+; Hyclone) for 1 h at room temperature. Buffer was removed and coverslips were incubated in a humidified chamber overnight at 4°C with either rabbit anti-CB_1 or rabbit anti-CB_2 receptor antibody (Affinity BioReagents, Golden, CO) diluted 1:500 in TBS+. Coverslips were washed in TBS+ before a 90-min darkened incubation with Alexa488-conjugated goat anti-rabbit antibody (Molecular Probes, Eugene, OR) diluted 1:1000 in TBS+. After washing with TBS, coverslips were mounted in Fluoromount G (Southern Biotechnology Associates, Birmingham, AL) onto labeled glass slides and visualized.

Flow Cytometry. For FACS analysis, 5×10^6 microglial cells were scraped in PBS from a culture flask. Cells were spun down and the pellet was resuspended in FACS buffer (PBS with 2% fetal bovine serum and 0.05% sodium azide). An equal amount of heat-inactivated rat serum was added to block nonspecific binding, and the cells were incubated 30 min on ice. After centrifugation, cells were resuspended in FACS buffer and incubated 30 min on ice with the primary antibody, rabbit anti-rCB_2 receptor. Samples were washed in the same buffer and incubated 30 min with Alexa 488-conjugated anti-rabbit secondary antibody diluted in FACS buffer, after which cells were washed twice and resuspended in PBS + 1% (w/v) paraformaldehyde. Cell staining was analyzed on a fluorescence-activated cell-sorter scan flow cytometer (BD Biosciences, San Jose, CA). Cells were gated to exclude the population nonspecifically stained with secondary antibody only, and percentage of gated cells determined using WinMDI.

Western Immunoblotting. To analyze levels of phosphorylated ERK, RTMGL1 cells were grown to approximately 60% confluence in 10-cm dishes. Growth medium was removed, cells were washed once in DMEM, and medium was replaced with DMEM for a 24-h starvation period. After 24 h, drugs were added in ≤1 μl ethanol. Ten minutes after adding drugs, 2.5% LADMAC-conditioned medium was added to cells to stimulate growth. An exception was the "starved" group of cells, which were allowed to grow an additional day and then starved with DMEM over the 24-h period during which other cells were stimulated with M-CSF and drug treatments. To make lysates, all cells were washed in PBS and scraped in 75 μl of M-PER extraction agent (Pierce, Rockford, IL) with protease inhibitor cocktail. Lysates were sonicated, and protein concentration was determined using the method of Bradford (1976). Equal amounts of protein were separated by electrophoresis on 10% SDS-polyacrylamide gels, and proteins were transferred to nitrocellulose. Blots were blocked in 5% milk made with PBS + 0.2% Tween 20 (PBST) and incubated overnight with a polyclonal antibody recognizing the phosphorylated form of p44/p42 MAPK (1:500 in PBST milk; New England Biolabs, Beverly, MA). Blots were washed and incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) before being incubated with enhanced chemiluminescence reagent

(Pierce) and exposed to film (Eastman-Kodak, Rochester, NY). To reprobe blots with primary antibody recognizing total p44/42 MAPK, blot was stripped in buffer (62.5 mM Tris-HCl, pH 6.7, 2% SDS, and 1 μl/ml β-mercaptoethanol) for 30 min. at 55°C. Blots were washed with PBST and reblocked in PBST milk before incubating in anti-total MAPK (1:500, Calbiochem, San Diego) and process completed as above. Band densities were determined using Image J, available through the National Institutes of Health website.

Statistical Analysis. For thymidine incorporation and LC-MS quantitated values, means with S.E. were determined for all collected samples. Values are expressed as a percentage of control. One-way ANOVA followed by post hoc Dunnett's multiple comparisons t test was used to determine statistical differences in proliferation and in endocannabinoid production between vehicle and treated cells. Bonferroni's multiple comparison post-test was used to determine statistical significance among treated groups. To determine significance among band densities, means with S.E. were found from three separate experiments, and one-way ANOVA followed by post-hoc Bonferroni's selected comparisons tests were used. All statistical values were calculated using Prism Software (GraphPad, San Diego, CA).

Results

Production of Endocannabinoids by RTMGL1 Microglia. The RTMGL1 microglial cells demonstrated robust conversion of [^{14}C]arachidonic acid to the endocannabinoid 2-AG, and a smaller amount of AEA production (Fig. 1A). 2-AG is shown as a double peak in the HPLC chromatogram because it isomerizes to 1-AG; unlike the other arachidonate metabolites isolated by our extraction method, neither peak was affected by addition of the cyclooxygenase inhibitor indomethacin (10 μM) or the lipoxygenase inhibitor nordihydroguaiaretic acid (10 μM; data not shown). Identities of both endocannabinoids were confirmed by LC-MS in selective ion monitoring mode using deuterated standards. Typical endocannabinoid contents were 400 ng of 2-AG (~1 nmol) and 1 ng of AEA (~3 pmol) per 5×10^6 microglial cells.

Synthesis Pathway of 2-AG in RTMGL1. To further demonstrate specificity of 2-AG production in RTMGL1 microglia, we examined its synthetic pathway. The immediate precursor of 2-AG is diacylglycerol (DAG), which is converted to 2-AG by DAG lipase (Stella et al., 1997). When pretreated with the DAG lipase inhibitor RHC 80267 (50 μM), RTMGL1 cells produced appreciably less 2-AG (~24% of control; Fig. 1B). There are two major putative pathways for cellular 2-AG synthesis, converging at DAG: one requiring hydrolysis of phosphatidylinositol and the other requiring hydrolysis of phosphatidic acid (Stella et al., 1997; Bisogno et al., 1999). We examined the effect of compounds inhibiting these two pathways and employed LC-MS for quantitation. A phospholipase C inhibitor (U73122, 1 μM) had no effect on microglial 2-AG production, whereas propranolol (100 μM), which inhibits phosphatidic acid phosphohydrolase, reduced the generation of 2-AG to approximately 35% of control (Fig. 1B).

Effect of Endocannabinoids on M-CSF–Dependent Microglial Proliferation. Using the RTMGL1 rat microglial cell line, we examined the effects of exogenously added endocannabinoids on M-CSF–dependent proliferation. This was done by assaying for incorporation of [^{3}H]thymidine into DNA/cellular macromolecules 24-h after stimulation of microglial cells with both exogenously added cannabinoid and M-CSF. Twenty-four hours after treatment, RTMGL1 exposed to the endocannabinoid 2-AG (500 nM) significantly ($p < 0.05$, Dunnett's test) increased microglial proliferation compared with vehicle-treated (control) cells (Fig. 2A). This proliferative effect was dependent upon the presence of M-CSF (data not shown) and was not seen with the endocannabinoid anandamide, even at concentrations up to 2 μM (Fig. 2B). We examined the effects of other cannabinoid agonists on RTMGL1 proliferation. The putative endogenous CB_1 receptor agonist noladin ether (Hanus et al., 2001) failed to increase proliferation at up to 200 nM, but the potent CB_2 receptor agonist JWH133 (100 nM) increased proliferation to

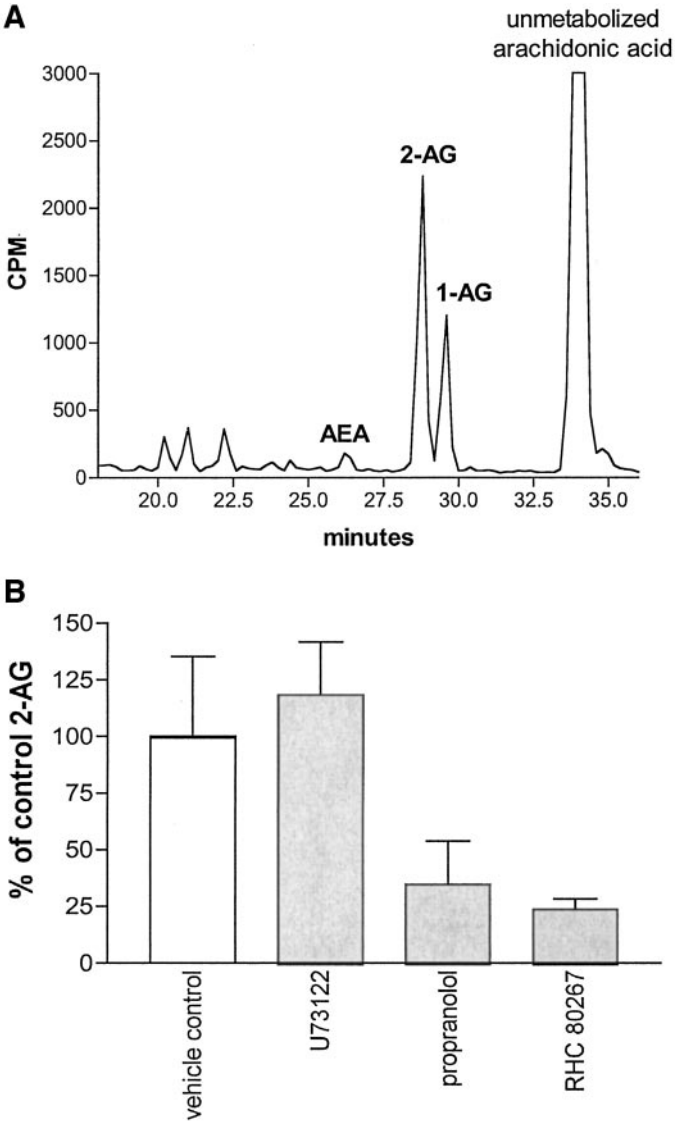

Fig. 1. RTMGL1 microglial cells produce the cannabinoids 2-AG and AEA; 2-AG synthesis occurs via conversion of phosphatidic acid. Cultured RTMGL1 cells were preincubated with [^{14}C]arachidonic acid in the presence of 10 μM A23187 ionophore. Lipids were extracted and metabolites of arachidonic acid measured by HPLC (A). Identity of 2-AG and AEA was confirmed by LC-MS in selective ion monitoring mode, using deuterated standards. To examine synthesis of 2-AG, RTMGL1 were incubated with 1 μM U73122, 100 μM propranolol, or 50 μM RHC 80267 for 10 min before incubation with arachidonic acid and ionophore. Lipids were extracted and amounts of 2-AG were quantified by LC-MS. Results are expressed as a percent of 2-AG produced by vehicle-treated control and are the means of two to six separate experiments. Error bars reflect S.E.M. Because of the variation in untreated control levels, the differences between the values were not found to be statistically significant by ANOVA test.

an extent similar to that of 2-AG (Fig. 2A). To further confirm receptor selectivity, effects of the high-affinity CB_1 receptor agonists arachidonylcyclopropylamide and arachidonylchloroethylamide were examined. These compounds failed to affect RTMGL1 proliferation at micromolar concentrations, as did the naturally occurring ethanolamide, palmitoylethanolamide (data not shown).

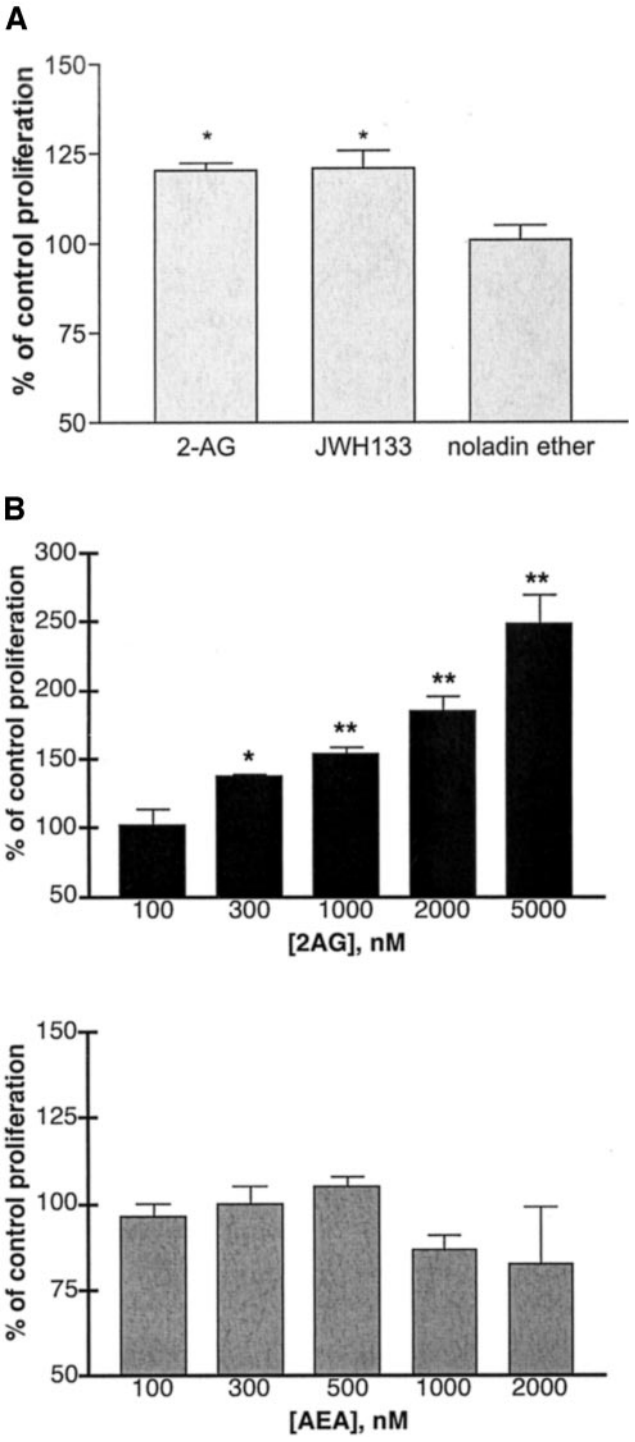

Fig. 2. RTMGL1 increase proliferation in the presence of exogenous 2-AG and the CB_2 receptor-selective agonist JWH133, but not the CB_1 receptor agonist noladin ether. Cultured RTMGL1 microglia were assayed for proliferation by measuring [^{3}H]thymidine incorporation as described under *Materials and Methods*. Drug treatments were added just before the final 24-h stimulation with CSF, and results expressed as percent of the mean vehicle-treated control thymidine incorporation. RTMGL1 were treated with 500 nM 2-AG, 100 nM JWH133, 200 nM noladin ether (A) or with increasing concentrations of 2-AG and AEA (B). Values are given in mean ± S.E.M., where n = 6 to 42 wells. *, $p < 0.05$; **, $p < 0.01$ significantly different from control (ANOVA followed by Dunnett's test).

Reversal of 2-AG–Stimulated RTMGL1 Proliferation by a CB_2 Receptor Inverse Agonist. SR144528 has been shown to act as a CB_2 receptor-selective inverse agonist/antagonist (Rinaldi-Carmona et al., 1998). To confirm that the proliferation induced by 2-AG and JWH133 was mediated by the CB_2 receptor, we examined RTMGL1 proliferation in the presence of SR144528 and with the specific CB_1 receptor antagonist SR141716. The proliferative responses to 2-AG were returned to control levels by the concurrent addition of SR144528; addition of SR141716 with 2-AG had no significant effect on cannabinoid-induced proliferation (Fig. 3). Administration of SR144528 alone had no effect on microglial proliferation. Because the CB_2 receptor is a G-protein coupled receptor (GPCR) whose effects are known to be mediated through the $G\alpha_i$ subunit (Bayewitch et al., 1995), we sought to demonstrate an inhibition of CB_2 receptor-mediated proliferation by concurrently treating cells with 100 ng/ml pertussis toxin, an irreversible $G\alpha_i/G\alpha_o$ inhibitor. However, normal M-CSF–dependent proliferation (i.e., in the absence of cannabinoids) was inhibited by pertussis toxin (33% of untreated control, data not shown). This is in agreement with data indicating that M-CSF–dependent proliferation requires functional $G_{i/o}$ protein (He et al., 1988; Hume and Denkins, 1989).

Molecular and Immunological Identification of CB_2 Receptor in RTMGL1 Microglial Cells. Our experiments indicate involvement of the CB_2 receptor in proliferation of RTMGL1 microglial cells in response to exogenously added cannabinoids. Through RT-PCR, immunofluorescence, and

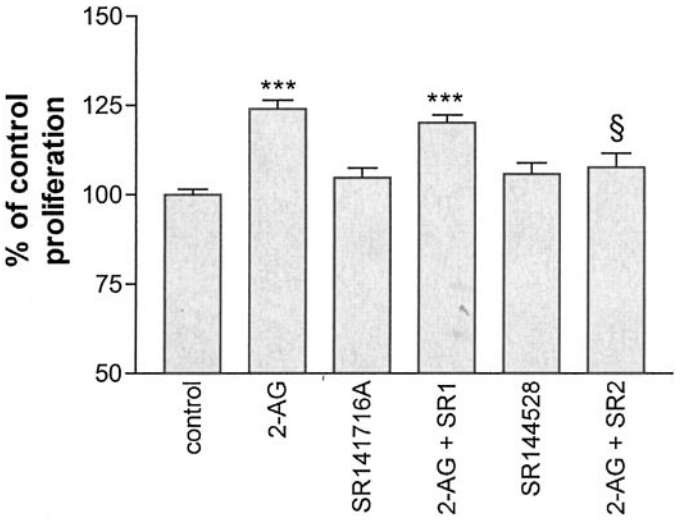

Fig. 3. Addition of the CB_2 receptor antagonist SR144528, but not the CB_1 receptor antagonist SR141716, abolished the RTMGL1 proliferative response. RTMGL1 microglia were assayed for proliferation by measuring [^{3}H]thymidine incorporation. RTMGL1 were treated with 500 nM 2-AG, 500 nM SR144528 (SR2), 500 nM SR141716 (SR1), or a combination of 500 nM 2-AG and 500 nM SR compound just before the final 24-h stimulation with M-CSF. All drug dilutions were made in ethanol and added in volumes of 0.4 to 0.6 μl. Results are expressed as a percentage of the ethanol-treated control, in mean ± S.E.M., where n = 18 wells. ***, $p < 0.001$ significantly different from control (ANOVA followed by Dunnett's test); §, $p > 0.05$ compared with control and $p < 0.001$ different from 2-AG treated alone (ANOVA, followed by Bonferroni's multiple comparison test).

FACS analysis, we confirmed the presence of the CB_2 receptor in these cells and also examined CB_1 receptor expression. RT-PCR using total mRNA isolated from RTMGL1 cells revealed transcript for the CB_2 receptor as well as mRNA for CB_1 receptor (Fig. 4A). Oligonucleotide primers to GAPDH were used as an amplification control, and RT-PCR reactions lacking reverse transcriptase served as a negative control for DNA contamination. Identification of receptor mRNA was confirmed through sequencing.

To examine cannabinoid receptor expression in individual RTMGL1 cells, immunofluorescence was performed using fixed, permeabilized cells. RTMGL1 expressed CB_2 receptor protein in a heterogeneous fashion throughout the cell body, apparent both perinuclear (Fig. 4B) and at the plasma membrane (Fig. 4B, inset). The CB_1 receptor was also expressed in these microglial cells (Fig. 4C).

We employed flow cytometry to examine cannabinoid receptor protein expression in the RTMGL1 microglial cell population. A nonpermeabilizing staining method was used with an antibody that recognizes the extracellular domain of CB_2 receptor to study receptor expression at the plasma membrane. Cells incubated with secondary antibody alone showed a basal level of fluorescence. When a primary antibody raised against the N terminus of the CB_2 receptor was added, a distinct population of CB_2 receptor-positive cells emerged (Fig. 4D and E). The percentage of microglial cells expressing CB_2 receptor at the cell surface remained constant at 50 to 60%, despite increased antibody concentration (data not shown). When RTMGL1 cells were treated for 24 h with 500 nM 2-AG, the population of CB_2 receptor-positive cells decreased to only 5%, suggesting receptor internalization or degradation (Fig. 4F). To examine possible CB_2 receptor degradation after 2-AG treatment, we costained treated, permeabilized RTMGL1 with CB_2 receptor antibody and DAPI, which stains cell nuclei. After a 24-h treatment with 500 nM 2-AG, every DAPI-stained cell also stained positive for CB_2 receptor; the intensity of CB_2 receptor staining was comparable with untreated microglia (data not shown). This suggests that the decrease seen in CB_2 receptor surface expression after 2-AG treatment is caused by internalization rather than degradation of available receptors.

Because the compound SR144528 acts as an inverse agonist at the CB_2 receptor and has been described to increase cell-surface CB_2 receptor expression in transfected CHO cells (Bouaboula et al., 1999), we examined its effects on RTMGL1 CB_2 receptor expression. However, a 24-h treatment with SR144528 failed to increase the number of microglia expressing a cell-surface CB_2 receptor (Fig. 4G), indicating that the compound does not increase receptor availability in RTMGL1 microglia.

Enhancement of ERK Activation in RTMGL1 Treated with 2-AG. Because both the CB_2 and the M-CSF receptors are known to signal through MAPK (Bouaboula et al., 1996; Brown et al., 2002), we examined activation of ERK1/2 (p44/p42 MAPK, respectively) in RTMGL1 cells. Overnight treatment with 2-AG modestly but significantly increased levels of phosphorylated, p44 MAPK in RTMGL1 cells, whereas total MAPK protein levels remained constant (Fig. 5). The increase in phospho-p44 MAPK was lowered to control levels when the CB_2 antagonist SR144528 was included in the 2-AG–treated culture, indicating that 2-AG is increasing MAPK activation via the CB_2 receptor. Because RTMGL1 starved of M-CSF have very low levels of active p44/p42 ERK (Fig. 5) and the mitogen-activated protein kinase kinase inhibitor PD98059 inhibits RTMGL1 proliferation (Rademacher et al., 2004), these data point to MAPK as a possible convergence point in M-CSF and CB_2 signaling to increase RTMGL1 proliferation.

Discussion

Endocannabinoid signaling is gaining recognition as a widely distributed and conserved system (Salzet et al., 2000; Elphick and Egertova, 2001). Both immune cells (Di Marzo et al., 1999) and neurons (Stella et al., 1997) have been shown to produce endocannabinoids. The first goal of this study was to examine the cellular production of two endocannabinoids in the M-CSF–dependent RTMGL1 rat microglial cell line. The second goal of these studies was to explore the role of cannabinoid receptor activity in RTMGL1 proliferation. Our results demonstrate that RTMGL1 microglial cells produce large amounts of the endocannabinoid 2-AG when stimulated and much smaller amounts of anandamide. When added exogenously to RTMGL1 cell cultures, 2-AG increases M-CSF–dependent cell proliferation, possibly through enhancement of ERK activity. Therefore, these studies add brain microglial cells to the growing list of immune cells that use endocannabinoids and cannabinoid receptors to modulate cellular function.

Other groups have demonstrated both 2-AG (Di Marzo et al., 1999) and AEA (Di Marzo et al., 1996; Schmid et al., 1997) production in rodent macrophages, which are functionally related to microglia. Similar to our results in microglia, AEA levels were very low in unstimulated J774 mouse macrophages (Di Marzo et al., 1999). More recently, murine microglia were shown to produce the endocannabinoids 2-AG and AEA, as well as the putative endocannabinoids homo-γ-linolenylethanolamide and docosatetraenylethanolamide, (Walter et al., 2003). This group found the predominant endocannabinoid in murine microglia to be AEA, at 13.6 pmol per milligram of protein (approximately two 10-cm dishes); however, they found levels of 2-AG increased 3-fold when microglia were incubated with a calcium ionophore (Walter et al., 2003). In the present studies, we also treated RTMGL1 with a calcium ionophore before measuring endocannabinoid production. Unstimulated RTMGL1 microglia produced basal amounts of endocannabinoids, and these levels increased 2- to 4-fold when ionophore was included. These findings are in accord with numerous reports that 2-AG and AEA are produced on demand in response to increases in intracellular calcium (reviewed in Hillard, 2000). Our data indicate that 2-AG synthesis in RTMGL1 microglial cells occurs via a phosphatidic acid precursor and is not affected by inhibition of phospholipase C. The specific mechanisms of microglial endocannabinoid production, as well as its physiological stimuli, remain unknown.

We have demonstrated that, among the endocannabinoids tested, 2-AG, but not AEA or noladin ether, exerted a proproliferative effect on RTMGL1. Structure/activity studies indicate that 2-AG is a full agonist at both CB_1 and CB_2 receptors, whereas anandamide is, at best, a weak partial agonist at the CB_2 receptor (Hillard et al., 1999; Gonsiorek et al., 2000). Similarly, noladin ether is not an agonist of the CB_2 receptor (Hanus et al., 2001). In addition, the 2-AG proliferative effect in RTMGL1 is mimicked by the CB_2 receptor-specific agonist JWH133 and blocked by the CB_2 re-

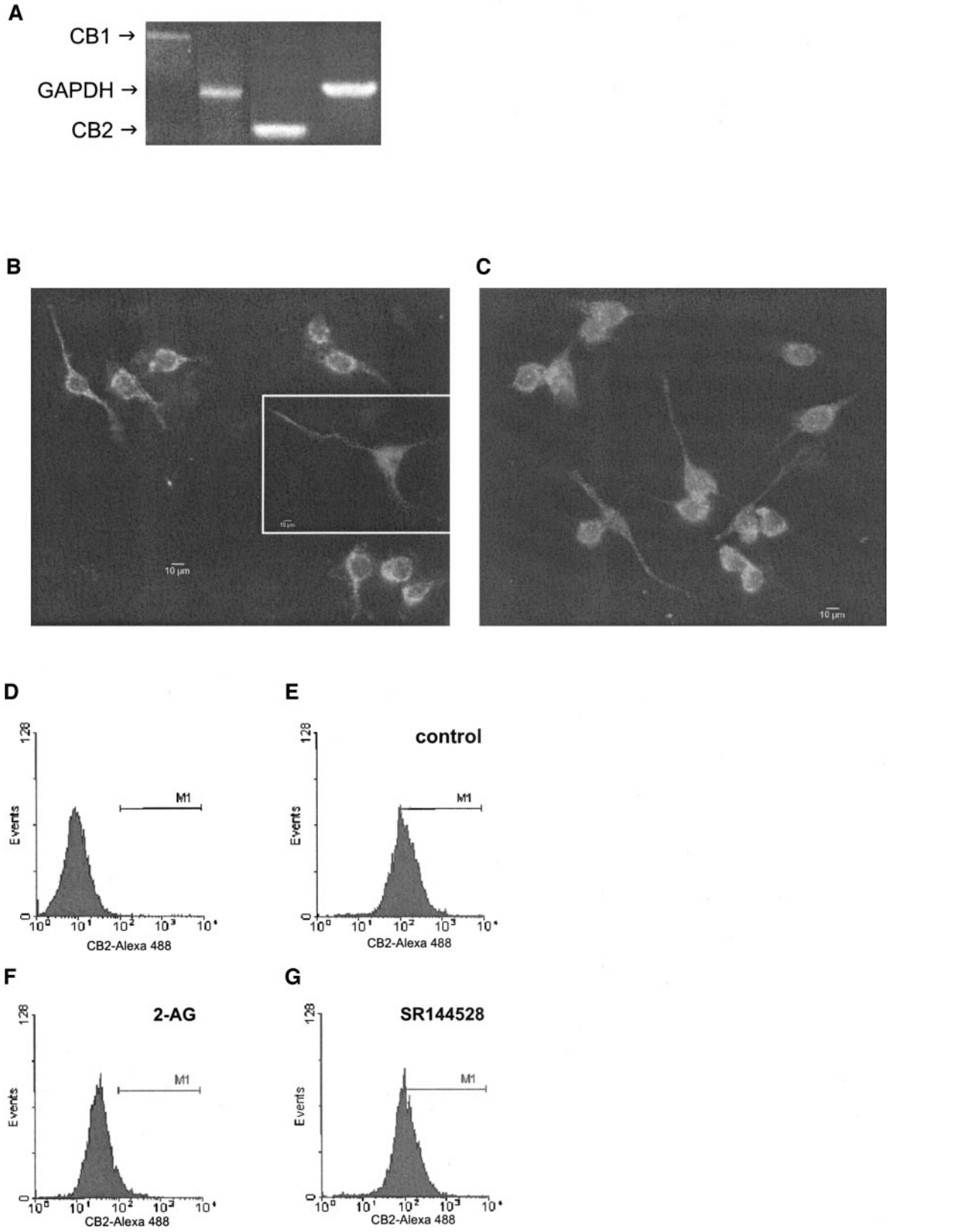

Fig. 4. RTMGL1 microglia express cannabinoid receptors. (A) mRNA was isolated from RTMGL1 cultures and RT-PCR was performed using primers developed against both CB_1 and CB_2 receptors. Primers against GAPDH were used as positive controls. B–C, RTMGL1 cells were examined for the presence of CB_2 (B) or CB_1 (C) receptor using specific primary antibodies and an Alexa 488-conjugated secondary antibody. D-G, histograms generated from FACS analysis of RTMGL1 cells using secondary anti-rabbit antibody only (D), or 1:100 dilution of anti-CB_2 receptor antibody, followed by Alexa 488-conjugated secondary (E–G). Cells were treated for 24 h with ethanol vehicle (E), 500 nM 2-AG (F), or 500 nM SR144528 (G) before staining for FACS analysis. To determine the population positive for a membrane CB_2 receptor, cells were gated for analysis (M1) based on the secondary-only histogram. Percentage of gated cells was as follows: 0.27% (D), 62% (E), 5.4% (F), and 48% (G).

ceptor antagonist SR144528, evidence that 2-AG induced proliferation is mediated by the CB_2 receptor. Because CB_2 receptor activation does not increase RTMGL1 proliferation in the absence of M-CSF, there is evidence for cross talk between the two receptors. The M-CSF receptor has been shown to communicate with pertussis toxin-sensitive GTP-binding proteins such as those activated by the CB_2 receptor (Hartmann et al., 1990). Similar enhancement of receptor tyrosine kinase signaling by a GPCR has been shown to transactivate other growth factor pathways, such as that of the epidermal growth factor receptor (Saito and Berk, 2001).

RTMGL1 microglia express cannabinoid receptor mRNA transcript, and display heterogeneous expression of CB_1 and CB_2 receptor protein. These data are in agreement with earlier reports regarding CB_1 and CB_2 receptor expression in primary and transformed microglial cells. It has been reported that rat (Sinha et al., 1998) and murine (Walter et al., 2003) microglia express the CB_1 receptor. Neonatal murine microglia also express the CB_2 receptor differentially in relation to cell activation state (Carlisle et al., 2002), with primed microglia expressing significant amounts of CB_2 receptor transcript but both resting and fully activated microglia expressing only small amounts of transcript. The M-CSF–dependent RTMGL1 rat microglial cells exist in a primed, proliferative state; therefore, given the previous findings, it is not surprising that they express the CB_2 receptor in functional amounts. Data generated by FACS analysis of nonpermeabilized RTMGL1 microglia indicate that almost 50% of microglia do not express CB_2 receptor at the cell surface, suggesting receptor internalization. Upon exposure to the agonist 2-AG, the microglial CB_2 receptor is almost completely internalized, which is further indication of a functional receptor.

Our findings, indicating that 2-AG and the CB_2 receptor-specific agonist JWH133 increase M-CSF-dependent proliferation of RTMGL1 microglia, are somewhat in conflict with the predominant anti-inflammatory effects of cannabinoids on the peripheral immune system. Some of these differences can be explained by the specific cannabinoids used; generally, the plant-derived cannabinoid Δ^9-THC has inhibitory effects on the immune system (Berdyshev, 2000), whereas the specific role of endocannabinoids in the immune system varies among cell types. Whereas AEA can reduce T- and B-lymphocyte proliferation when added alone (Schwarz et al., 1994), AEA stimulates the proliferation of numerous murine myeloid cell lines when added in conjunction with either interleukin-3 or M-CSF (Valk et al., 1997). Other pro-inflammatory evidence comes from data demonstrating that both 2-AG and AEA increase migration of BV-2 murine microglial cells (Walter et al., 2003).

We demonstrate that stimulation of the CB_2 receptor results in possible receptor internalization, as well as increased phosphorylation of p44 MAPK and proliferation (Rademacher et al., 2004). The time course of the events leading to proliferation is prolonged, as ERK1 activation, proliferation, and receptor internalization were demonstrated a full 24 h after exogenous 2-AG was added. This sustained ERK1 activation seems to be necessary for proliferation, because a shorter, 4-h incubation with 2-AG has no effect on RTMGL1 proliferation (data not shown). Because quantification through LC/MS has shown the half-life of exogenous 2-AG in RTMGL1 cultures to be only 2 to 3 min (data not shown) because of rapid metabolism, the prolonged time course is not a result of sustained receptor activation. Likewise, because proliferation and ERK1 activation were both blocked with the CB_2 receptor antagonist SR144528, these events are probably not caused by an accumulation of arachidonic acid from 2-AG degradation. Although the full mechanism is unknown, this brief activation of CB_2 receptors resulting from 2-AG addition seems to initiate a prolonged cascade resulting in increased ERK1 activation and eventual proliferation.

Because 2-AG is produced by the RTMGL1 microglial cells and also functions to increase cell proliferation in conjunction with M-CSF, it is possible that microglial cells produce 2-AG as an autacoid costimulatory molecule. However, there is

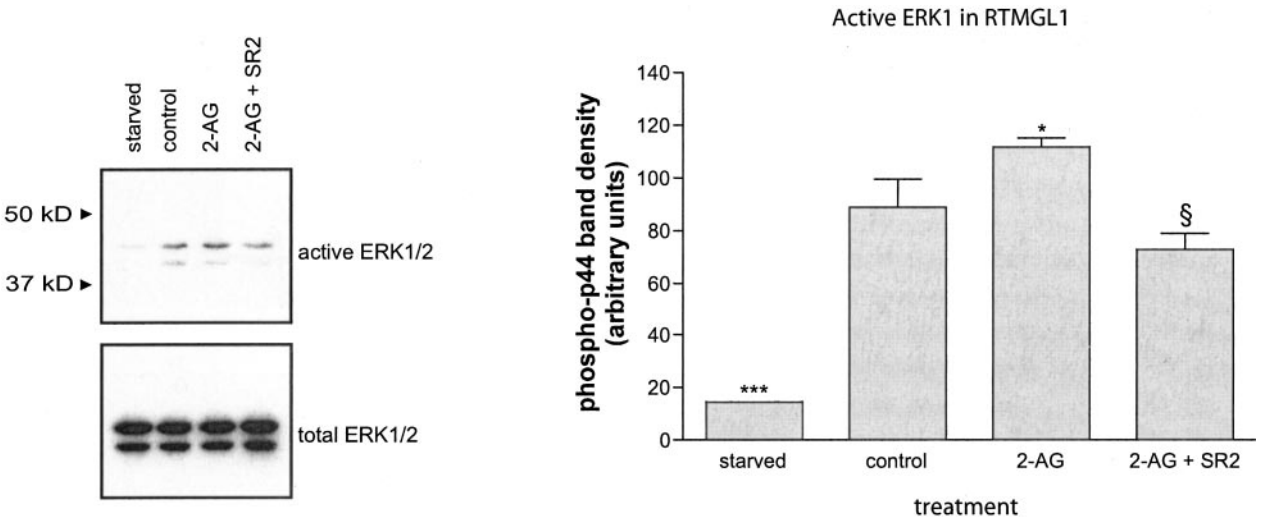

Fig. 5. 2-AG activation of the CB_2 receptor increases active ERK1 in RTMGL1. RTMGL1 were grown, starved, and treated 20 h with vehicle, 500 nM 2-AG, or 500 nM SR144528 (SR2) and stimulated by addition of M-CSF as in proliferation assays. "Starved" cells were grown in parallel with the other cultures but denied M-CSF for 24 h before lysing. Equal amounts of protein were resolved by SDS-PAGE, and the results were evaluated by Western blotting using a phosphospecific anti-ERK1/2 antibody (upper left). Blot was stripped and reprobed with an antibody recognizing total ERK1/2 (lower left). Shown is one of three experiments. Results at right are means ± S.E.M. of p44 band densities ($n = 3$). *, $p < 0.05$; ***, $p < 0.001$ significantly different from control; §, $p > 0.05$ different from control and $p < 0.01$ different from 2-AG treated alone (ANOVA followed by post-hoc Bonferroni's selected comparisons tests).

evidence that 2-AG does not serve as an autacoid in unstimulated microglia. First, if 2-AG were tonically produced as a proliferative factor, we would expect addition of the CB_2 receptor inverse agonist/antagonist SR144528 alone to have negative effects on microglial proliferation. Contrary to this prediction, we have observed no significant difference in proliferation between SR144528-treated cells and control cells. In agreement with these observations, neither a CB_1 nor a CB_2 receptor inverse agonist reduced baseline levels of microglial migration in BV-2 microglia (Walter et al., 2003), which also produce 2-AG. Whereas the CB_2 receptor inverse agonist SR144528 has been shown to increase the number of CB_2 receptors available for binding at the cell surface, theoretically leading to enhanced agonist sensitivity (Bouaboula et al., 1999), our FACS data do not show any increase in cell surface RTMGL1 CB_2 receptors upon addition of SR144528. Therefore, although 2-AG is produced by microglia at rest, our data indicate that the endocannabinoid does not function through the CB_2 receptor in unstimulated cells.

Microglial proliferation and activation has been tied to many chronic neurodegenerative diseases, including multiple sclerosis, Alzheimer's, and AIDS-associated dementia (Liu and Hong, 2003). Our study shows that the RTMGL1 rat microglial cell line produces the endocannabinoid 2-AG, which acts through the CB_2 receptor to enhance CSF-dependent proliferation. Further study regarding CB_2 receptor-stimulated microglial proliferation in neurodegenerative disease models could yield a specific target in preventing neuronal destruction by reactive microglia.

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