Effects of Peripheral Cannabinoid Receptor Ligands on Motility and Polarization in Neutrophil-like HL60 Cells and Human Neutrophils**

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The possible role of the peripheral cannabinoid receptor (CB2) in neutrophil migration was investigated by using human promyelo-
cytic HL60 cells differentiated into neutrophil-like cells and human neutrophils isolated from whole blood. Cell surface expression of CB2 on HL60 cells, on neutrophil-like HL60 cells, and on human neutrophils was confirmed by flow cytometry. Upon stimulation with either of the CB2 ligands JWH015 and 2-arachidonoylglycerol (2-AG), neutrophil-like HL60 cells rapidly extended and retracted one or more pseudopods containing F-actin in different directions instead of developing front/rear polarity typically exhibited by migrating leukocytes. Activity of the Rho-GTPase RhoA decreased in response to CB2 stimulation, whereas Rac1, Rac2, and Cdc42 activity increased. Moreover, treatment of cells with RhoA-dependent protein kinase (p160-ROCK) inhibitor Y27632 yielded cytoskeletal organization similar to that of CB2-stimulated cells. In human neutrophils, neither JWH015 nor 2-AG induced motility or morphologic alterations. However, pretreatment of neutrophils with these ligands disrupted N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP)-induced front/rear polarization and migration and also substantially suppressed fMLP-induced RhoA activity. These results suggest that CB2 might play a role in regulating excessive inflammatory response by controlling RhoA activation, thereby suppressing neutrophil migration.

The peripheral cannabinoid receptor (CB2)† was cloned in 1993 (1) after cloning of the central cannabinoid receptor (CB1) in 1990 (2). It has been suggested that the gene encoding CB2 is a protooncogene and that aberrant expression of CB2 in myeloid precursor cells results in the development of leukemia by blocking neutrophil differentiation (3, 4). CB2 is expressed predominantly in immune cells (5), and because of the diversity of immune cells, it is assumed that CB2 is involved in various activities in addition to inhibition of neutrophil differentiation (6–9). Steffens et al. (10) recently reported that doses of Δ⁹-tetrahydrocannabinol (the most psychoactive component of marijuana) too low to have psychotropic effects inhibit the progression of atherosclerosis via immunomodulatory effects on lymphoid and myeloid cells. This report indicates that CB2 may be involved in a wide range of physiologic phenomena related to immunity and that some CB2 ligands may have application in the treatment of inflammatory disease. However, research into CB2 is still in its early stages. In particular, the involvement of only a few molecules, Gαi2/Gαi3, protein, phosphatidylinositol 3-kinase (PI3K), and members of the mitogen-activated protein kinase and nuclear factor-κB families, in the CB2 signaling pathways has been reported (6–8, 11).

Among the possible roles of CB2 in immunity is the induction of leukocyte migration to sites of infection and inflammation, an important step in the host defense against pathogenic microorganisms. CB2 is a seven-transmembrane, Gαi2/Gαi3-protein-coupled receptor, as are receptors for chemoattractants such as N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP). It has been reported that several genes encoding chemotactic cytokines are up-regulated in response to CB2 stimulation (12). Indeed, 2-arachidonoylglycerol (2-AG), a physiological ligand for both CB1 and CB2 (13, 14), can induce migration in some subpopulations of hematopoietic cells (8, 15, 16). However, contradictory findings have been reported by other researchers (10, 17–19), in part due to differences in ligands and cells used. In particular, Steffens et al. (10) reported that Δ⁹-tetrahydrocannabinol inhibits monocyte chemoattractant protein 1-induced macrophage migration, a crucial step in the progression of atherosclerosis.

In the present study, the possible role of CB2 in neutrophil migration was investigated. We constructed an in vitro model of neutrophil migration on blood vessels as described previously (20) but with some modification: we studied human promyelocytic HL60 cells, differentiated by all-trans-retinoic acid (ATRA) into neutrophil-like cells, on plates coated with fibrinogen, an adhesive extracellular matrix glycoprotein. We show that two CB2 ligands, 2-AG and a synthetic CB2-specific ligand JWH015, induce increased motility of the cells but that the cells do not develop the front/rear polarity that migrating leukocytes typically exhibit, with a lamellipodium at the front and a retracting tail at the rear (21, 22).

In addition, to investigate the possible physiological implication of the results, the effects of the CB2 ligands, alone and as pretreatment before fMLP stimulation, were analyzed in human neutrophils isolated from whole blood. We show that pretreatment of human neutrophils with the ligands disrupts fMLP-induced front/rear polarization and migration.
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**EXPERIMENTAL PROCEDURES**

Antibodies and Reagents — The rabbit polyclonal anti-CB2 antibody was purchased from Calbiochem Corp. The mouse monoclonal anti-GFP antibody (sc-9996) and the rabbit polyclonal anti-RhoA (sc-179) and anti-Rac2 (sc-96) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). The mouse monoclonal anti-α-tubulin antibody was from Sigma. The mouse monoclonal anti-Rac1 and anti-Cdc42 antibodies were from BD Biosciences. The mouse monoclonal anti-phosphorylated (serine 473) PKB/Akt, the rabbit polyclonal anti-PKB/Akt, the mouse monoclonal anti-phosphorylated myosin light chain (MLC) II, and the rabbit polyclonal anti-MLC II antibodies were from Cell Signaling Technology (Beverly, MA). The Alexa Fluor 488-conjugated goat secondary anti-mouse and anti-rabbit antibodies were from Invitrogen Corp. The HRP-conjugated goat secondary anti-mouse and anti-rabbit antibodies were from Bio-Rad. Alexa Fluor 594-conjugated phalloidin was from Invitrogen Corp. Human fibrinogen was from Yoshitomi-yakuhin Corp. (Osaka, Japan). JWH015 and 2-AG were from Biomol Research Laboratories (Plymouth Meeting, PA). CB2 inhibitor AM251 was from Tocris Bioscience (Ellisville, MO). fMLP was purchased from Calbiochem Corp. The mouse monoclonal anti-Rac1 and anti-Cdc42 antibodies were from Invitrogen Corp. Human fibrinogen was from Yoshitomi-yakuhin Corp. (Osaka, Japan). JWH015 and 2-AG were from Biomol Research Laboratories (Plymouth Meeting, PA). CB2 inhibitor AM251 was from Tocris Bioscience (Ellisville, MO). fMLP was from Sigma. Y27632 was from Calbiochem Corp. in

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**Experiment H**

Plasmid Constructs and DNA Transfection — The PH domain of human pkb/akt protein kinase (amino acids 1–167) was amplified from human spleen CDNA (Ambion, Austin, TX) with the following primer pair: 5'-ATGAGGCACGGTTGCTATGTTGAAGG-3' and 5'-CACGAGGATCACCTGGCCAAAGTG-3'. The second amplification reaction was performed with primers that contained EcoRI and BamHI restriction sites and the amplified products were cloned into the pEGFP-N1 plasmids (Clontech Laboratories, Inc.). After the sequences of both strands were verified with ABI PRISM Cycle Sequencing FS Ready Reaction Kit (Applied Biosystems, Foster City, CA), the plasmids were transfected into COS-7 cells. Cell lysates were resolved by SDS-PAGE, and subjected to immunoblot analysis for the presence of GFP fusion proteins with anti-GFP antibody. GFP-PKB/Akt-PH domain was introduced into HL60 cells by electroporation.

**Cell Migration Assay** — Migration of live cells was observed as described previously (23) but with some modification. In brief, cells were plated in culture medium onto a 100 μg/ml fibrinogen- or 10 μg/ml fibrinectin-coated 35-mm culture dish, which was placed on the stage of a Power IX microscope (Olympus Corp., Tokyo, Japan) coupled to an MI-LBC CO2 incubator (Olympus Corp.) to allow for observations at 37 °C in 95% air and 5% CO2. Cells were stimulated with either uniform concentration of ligands or a point source of ligands from a micropipette (Femtotips®; Eppendorf AG, Hamburg, Germany). Cell morphology and motility alterations were monitored by obtaining single-frame images every 10 s for at least 20 min. Approximately 160–200 cells were present per 297.0 × 397.9-μm frame. Those cells which remained in the frame throughout the observation (~90–95%) were examined. Cell lengths and migratory distances were measured with image analyzing software MacSCOPE Version 2.6 (Mitani Corp., Fukui, Japan). In particular, migratory distances were calculated by monitoring two-dimensional coordinates of each cell center at 30-s intervals. Other morphologic alterations were examined by observing individual cells during stop-time playback of time-lapse images.

**Immunofluorescence Microscopic Analysis** — In preparation for staining, cells were plated in culture medium onto 100 μg/ml fibrinogen-coated FALCONTM culture slides (BD Biosciences), fixed with 3% paraformaldehyde for 15 min, washed twice with PBS containing 2 mM MgCl2 and 0.5% BSA (staining buffer), and permeabilized with 0.2% Triton X-100 in staining buffer for 3 min. Nonspecific binding to Fc receptors was blocked by incubation for 15 min with PBS containing 2 mM MgCl2 and 5% BSA. To confirm CB2 expression, samples thus prepared were incubated with Alexa Fluor 488-conjugated anti-CB2 antibody for 45 min. This antibody was produced with the use of a ZenontM rabbit IgG labeling kit (Invitrogen Corp.) according to the manufacturer’s instructions. After three washes with staining buffer, chromosomes were stained with the DNA-specific fluorescent dye Hoechst 33342 (Wako Pure Chemical Industries, Osaka, Japan). To examine cytoskeletal organization, prepared cells were incubated with anti-α-tubulin antibody for 45 min. After three washes with staining buffer, cells were incubated with Alexa Fluor 488-conjugated secondary anti-mouse antibody and Alexa Fluor 594-conjugated phalloidin (to visualize F-actin) for 30 min. To examine localization of the active form of MLC II, cells were incubated with anti-phosphorylated MLC II antibody. Following procedures were identical to those for staining of α-tubulin. All of these procedures were conducted at room temperature.

Stained cells were washed twice with staining buffer, mounted in antifade mounting medium (ProLongTM Antifade kit; Invitrogen Corp.), and analyzed by confocal microscopy with an LSM 510 laser scanning unit and an Axiovert 200 M inverted microscope (Carl Zeiss, Oberkoch, Germany) run with imaging software LSM 510 META Version 3.0 (Olympus Corp.).

**Rho-GTPase Pull-down Assay** — The GTP-bound (active) forms of four Rho-GTPases, Rac1, Rac2, Cdc42, and RhoA, were isolated by pull-down assay. Recombinant protein PAKI-PBD-GST, which binds specifically to the GTP-bound forms of Rac and Cdc42, was produced as described previously (24). In brief, cDNA of the Rac- and Cdc42-binding domain of human PAKI (PAKI-PBD; amino acids 67–150) was cloned into expression vector pGEX4T-3 as a fusion protein with glutathione S-transferase (GST) and was expressed in Escherichia coli DH5α cells treated with isopropyl β-D-thiogalactopyranoside (Nacalai Tesque, Kyoto, Japan). The fusion protein was purified with glutathione-Sepharose 4B beads (Amersham Biosciences, Uppsala, Sweden). For production of recombinant protein rhotekin-RBD-GST, which binds specifically to the GTP-bound forms of Rho (-A, -B, and -C), cDNA of the Rho-binding domain of mouse rhotekin (rhotekin-RBD;
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Neutrophil-like HL60 cells and human neutrophils (1 × 10⁷) were stimulated with 100 nM JWH015, 300 nM 2-AG, or 100 nM fMLP. Because cell adhesion to extracellular matrix affects Rho-GTPase activity (25) and because cells are known to polarize in suspension culture (26), we stimulated cells in suspension culture in a BSA-coated microtube. The cells were then lysed for 15 min at 4 °C in Mg²⁺-lys buffer (MLB) containing 100 mM NaCl, 50 mM Tris-HCl (pH 7.5), 1% Triton X-100, 10 mM MgCl₂, 1 mM phenylmethylsulfonfyl fluoride, 1 mM Na₃VO₄, and 1.5 mM aprotinin. Cell lysates were centrifuged at 12,000 × g for 15 min at 4 °C. A proportion of each supernatant was diluted in Laemmli sample buffer at 100 °C for 5 min for detection of total (both GTP- and GDP-bound) Rho-GTPases. The remaining supernatants were incubated with either PAK1-PBD-GST or Rho-GTPases. The remaining supernatants were incubated with either PAK1-PBD-GST or PAK1-PBD-GST for 1 h at 4 °C followed by three washes with MLB. Proteins bound to the beads were eluted by being heated in Laemmli sample buffer at 100 °C for 5 min and, along with samples for detecting total Rho-GTPases, were subjected to Western blot analysis.

Western Blot Analysis—To detect activity of the four Rho-GTPases, we used samples lysed in MLB. To detect PAK/Btk and MLC II activity, cells were lysed in a solution containing 100 mM NaCl, 50 mM Tris-HCl (pH 7.2), 1% EDTA, 1% Triton X-100, 1 mM phenylmethylsulfonfyl fluoride, 2 mM Na₃VO₄, and 1.5 mM aprotinin. Proteins were separated by SDS-PAGE and transferred to polyvinylidene fluoride microporous membrane (Millipore Corp., Bedford, MA). The membranes were then blocked with 1% skim milk in PBS for 30 min at room temperature, incubated with antibodies to Rac1, Rac2, Cdc42, RhoA, phosphorylated PAK/Btk, or phosphorylated MLC II for 1 h at room temperature, and washed three times with a solution containing 150 mM NaCl, 25 mM Tris-HCl (pH 8.0), and 0.1% Tween 20 (TBS-T). The membranes were then incubated with HRP-conjugated secondary anti-mouse antibody (to detect Rac1, Cdc42, phosphorylated PAK/Btk, or phosphorylated MLC II) or HRP-conjugated secondary anti-rabbit antibody (to detect RhoA and Rac2) for 30 min at room temperature and washed twice with TBS-T and once with PBS. Immune complexes were visualized with Western Lightning™ chemiluminescence reagent (PerkinElmer Life Sciences) and a lumino-image analyzer (LAS-1000; Fuji Photo Film, Tokyo, Japan). For quantification, the density of each band was measured with image analyzing software NIH Image Version 1.63 f.

To detect total (both phosphorylated and unphosphorylated) PAK/Btk and MLC II, immune complex of HRP-conjugated secondary anti-mouse antibody with anti-phosphorylated PAK/Btk and that with anti-phosphorylated MLC II antibody were stripped by bathing the membranes in a solution containing 62.5 mM Tris-HCl (pH 6.8), 7 mM SDS, and 95 mM 2-mercaptoethanol for 30 min at 50 °C, and the membranes were reprobed with either general anti-PAK/Btk or anti-MLC II antibody.

Statistical Analysis—Statistical significance was determined by Student’s t test, with a p value of <0.05 considered significant.

RESULTS

CB2 Was Detected on the Surface of Neutrophil-like HL60 Cells and Human Neutrophils—HL60 cells are known to differentiate morphologically and functionally into neutrophil-like cells when treated with ATRA (27). We examined CB2 expression in HL60 cells, in HL60 cells 4 days after exposure to 1 μM ATRA (ATRA-treated HL60 cells), and in human PMNs isolated from whole blood. By flow cytometric analysis, CB2 was detected on the surface of these cells (Fig. 1A, panels a–c). The cell surface expression levels of CB2, as reflected by fluorescence intensity, were highest in HL60 cells; ATRA-treated HL60 cells and PMNs showed levels similar to each other (Fig. 1A, panel d). Prior to flow cytometric analysis, we confirmed by May-Grunwald-Giemsa staining that 77 ± 3% of ATRA-treated HL60 cells revealed neutrophilic maturation, and >95% of the PMNs were mature neutrophils (data not shown). Moreover, CB2 expression in these cells was confirmed by immunofluorescence microscopic analysis (Fig. 1B, panels d–f). We also observed nuclear morphology with Hoechst 33342 and confirmed that ATRA-treated HL60 cells were successfully induced to differentiate into neutrophil-like cells (Fig. 1B, panel b), and that most of the PMNs were mature neutrophils (Fig. 1B, panel c). Because similar levels of CB2 expression in neutrophil-like HL60 cells and in human neutrophils were confirmed, we used neutrophil-like HL60 cells as an in vitro model of human neutrophils in further experiments.

JWH015 and 2-AG Induced Motility with No Front/Rear Polarization in Neutrophil-like HL60 Cells—Alterations in motility and morphology of neutrophil-like HL60 cells following stimulation with either 100 nM JWH015 (a synthetic CB2-specific ligand) or 300 nM 2-AG (a physiological ligand for both CB1 and CB2) were monitored by video microscopy. Most unstimulated cells were spherical, with occasional small spike-like projections (Fig. 2A, upper left image). Approximately 3 min after JWH015 or 2-AG stimulation, 60% of the cells elongated (i.e., polarized), rapidly extending and retracting one or more pseudopods in different directions (Fig. 2A (JWH015); data not shown for 2-AG). These cells did not develop the front/rear polarity that migrating leukocytes typically exhibit and displayed almost no migratory activity during observations of up to 60 min (data not shown). A point source of 10 μM JWH015 by micropipette also induced polarization accompanied by the extension of one or more pseudopods in different directions (Fig. 2B). Quantitative analysis of image sequences of samples unstimulated or stimulated with uniform concentration of the CB2 ligands is shown in Fig. 2, C–E. To ensure consistency in distinguishing polarized cells from non-responding cells, cells with a value of X greater than 2 were counted as polarized (X = L/W, where L is the longest distance across the cell, and W is the greatest width perpendicular to L as shown in the illustration next to Fig. 2C). The percentage of polarized cells increased significantly after stimulation with the CB2 ligands (p < 0.01). This increase was almost completely inhibited by pretreatment with 1 μM CB2 inhibitor SR144528 but not by 1 μM CB1 inhibitor AM251 (Fig. 2C). In addition, the percentage of cells that extended multiple pseudopods increased significantly after CB2 stimulation (p < 0.01, Fig. 2D). Approximately 90% of the cells that polarized in response to the CB2 ligands were devoid of a retracting tail of the sort observed at the rear of migrating leukocytes (Fig. 2E). In contrast, >90% of the cells responding to uniform concentration (100 nM) of fMLP exhibited front/rear polarity, with a single pseudopod (lamellipodium) at the front and a retracting tail at the rear (Fig. 2, D and E; an image of a typical fMLP-stimulated cell is shown in the illustration next to Fig. 2E).

F-actin Formed in the Pseudopods in CB2-stimulated Cells—The cytoskeletal reorganization underlying the morphologic alterations accompanying CB2 stimulation was investigated by immunofluorescence microscopy. Fig. 3A, panels a–d, shows fluorescence images of F-actin (red) and microtubules (green). In a typical unstimulated cell (panel a), only faint F-actin staining was observed at the periphery, and from a single point, an array of microtubule filaments extended radially to the cell periphery. Three types of JWH015-stimulated cells are shown. In one type with a single pseudopod (panel b), a large accumulation of F-actin was observed in the pseudopod, toward which microtubule filaments emanated from a single point. In the other types, F-ac-
FIGURE 1. CB2 expression in human promyelocytic HL60 cells, in ATRA-treated HL60 cells, and in human PMNs. HL60 cells were induced to differentiate into neutrophil-like cells by treatment with 1 μM ATRA for 4 days (ATRA-treated cells). PMNs were isolated from human whole blood. A, flow cytometric analysis of cell surface expression of CB2 on HL60 cells, on ATRA-treated HL60 cells, and on PMNs. The histograms (panels a–c) are representatives of each experiment. The x axis represents the fluorescence intensity and the y axis the number of cells. Filled areas represent CB2 expression, and non-filled areas represent background fluorescence intensity. The bar graph (panel d) shows the relative fluorescence intensities normalized to the mean fluorescence intensity of untreated HL60 cells. Results are based on the mean values and S.D. of three independent experiments. *, p < 0.05. B, immunofluorescence microscopic analysis of CB2 expression in HL60 cells, in ATRA-treated HL60 cells, and in PMNs. Nuclear morphology was analyzed with Hoechst 33342. Columns: left, HL60 cells; middle, ATRA-treated HL60 cells; and right, PMNs. Panels a–c, images of cell nuclei. Panels d–f, images of CB2. Panels g–i, differential interference contrast images. Scale bar: 5 μm.
tin was found in two (panel c) or 3 (panel d) pseudopods, and microtubule filaments emanated from a single point to each pseudopod.

GFP-PKB/Akt-PH Domain Accumulated in One or More Regions in Neutrophil-like HL60 Cells—HL60 cells transiently expressing GFP-PKB/Akt-PH domain, a fluorescence probe for phosphatidylinositol 3,4,5-triphosphate and other products of PI3K (PI3Ps) (28, 29), were induced to differentiate into neutrophil-like cells by treatment with ATRA and stimulated with JWH015. Distribution of GFP-PKB/Akt-PH domain was examined by immunofluorescence microscopy. In cells that exhibited typical morphologic alterations in response to CB2 stimulation, GFP-PKB/Akt-PH domain accumulated in one or more regions, mostly in pseudopods (Fig. 3B, panels b and c).

JWH015 Decreased RhoA Activity in Neutrophil-Like HL60 Cells—Among numerous molecules implicated in cytoskeletal reorganization and cell migration, four Rho-GTPases, RhoA, Rac1, Rac2, and Cdc42, play particularly important roles. The effects of CB2 stimulation on the activities of these four Rho-GTPases were assessed by Rho-GTP pull-down assay, where active (GTP-bound) forms of Rho-GTPases can be
JWH015 and 2-AG Disrupted FMLP-induced Front/Rear Polarization and Migration of Human Neutrophils—Human neutrophils were stimulated with either 100 nM JWH015 or 300 nM 2-AG and subjected to cell migration assays. Neutrophils showed no motility or morphologic alterations in response to either ligand (Fig. 6A, panels b and c), whereas Rac1 and Rac2 activity showed an increase in response to CB2 stimulation. The CB2 ligand-induced increase in Rac1 and Rac2 activities was suppressed by CB2 inhibitor SR144528 (Fig. 6, B and C). These results suggest that CB2 receptors do function and that Rac1 and Rac2 activation does not necessarily lead to motility and morphologic alterations in human neutrophils.

In contrast to the lack of motility and morphologic alterations in response to the CB2 ligands, more than 70% of neutrophils responded to 100 nM fMLP; they polarized, developed front/rear polarity, and began to migrate 5 min after fMLP stimulation (Fig. 6A, panel d).

The effects of pretreatment with JWH015 or 2-AG on fMLP-induced front/rear polarization and migration were then investigated. Pretreated cells also exhibited motility and morphologic alterations in response to fMLP. However, most did not develop distinct front/rear polarity. Instead, they extended one or more pseudopods in different directions (Fig. 6A, panels e and f). The migratory velocity, which was calculated from the migratory distance of each cell (excluding nonresponding cells) from 5 to 20 min after fMLP stimulation, was significantly reduced by JWH015 or 2-AG pretreatment for 2 min (p < 0.01). This suppressive effect of 2-AG, a ligand for both CB1 and CB2, on fMLP-induced neutrophil migration was substantially countered by CB2 inhibitor SR144528 but not by CB1 inhibitor AM251. A similar effect of SR144528 was observed with JWH015 pretreatment (Fig. 6D).

In neutrophil-like HL60 cells, JWH015 stimulation decreased RhoA activity as described above (Fig. 4A). In addition, the effects on RhoA activity of the two CB2 ligands, alone and as pretreatment before fMLP stimulation, were assessed in human neutrophils. Whereas stimulation with JWH015 for 2 min induced no significant change in RhoA activity, stimulation with 2-AG for 2 min decreased RhoA activity. Stimulation with fMLP for 2 min increased RhoA activity. Pretreatment with both JWH015 and 2-AG for 2 min substantially suppressed the increase in RhoA activity induced by fMLP (Fig. 6E). In contrast, neither ligand affected the percentage of polarized cells in response to fMLP (Fig. 6F).

**DISCUSSION**

Upon stimulation with chemoattractants such as fMLP, leukocytes change rapidly from a spherical to an elongated shape with front/rear polarity and migrate within minutes. It was decided that capturing these rapid alterations by direct observation of cells over a short period following CB2 stimulation would be an aid in investigating the role of CB2 in neutrophil migration. Cells were monitored by time-lapse video microscopy every 10 s for 20–60 min after exposure to the CB2 ligands. We found that both JWH015 and 2-AG induced motility and previously unobserved morphologic alterations (the extension of one or more pseudopods) in neutrophil-like HL60 cells (Fig. 2A). The CB2 ligand-induced alterations were almost completely inhibited by pretreatment with CB2 inhibitor SR144528 but not by CB1 inhibitor AM251 (Fig. 2C), suggesting that the alterations specifically involve CB2. In addition, GFP-PKB/Akt-PH domain, which is known to localize at the front of migrating leukocytes (29, 30), accumulated in one or more pseudopods in CB2-stimulated cells (Fig. 3B, panels b and c). This result indicates that CB2 does not induce front/rear polarization. In contrast, cells responding to fMLP developed front/rear polarity (Fig. 2, D and E).
FIGURE 4. Changes in RhoA, Rac1, Rac2, Cdc42, and PKB/Akt activity levels in neutrophil-like HL60 cells in response to JWH015. The GTP-bound forms of four Rho-GTPases, RhoA, Rac1, Rac2 and Cdc42, were isolated from neutrophil-like HL60 cells by pull-down assay and, along with total (both GTP- and GDP-bound) Rho-GTPases, were analyzed by Western blot. Phosphorylated PKB/Akt and total (both phosphorylated and unphosphorylated) PKB/Akt were detected by Western blot with anti-phospho-PKB/Akt and anti-PKB/Akt antibodies, respectively. The figure shows the time courses of RhoA (A), Rac1 (B), Rac2 (C), Cdc42 (D), and PKB/Akt activity (E). The immunoblot images shown are representative of each experiment. Upper images show the intensities of active forms, and lower images show the intensities of total Rho-GTPases or PKB/Akt in samples at 0 s (lane 1), 30 s (lane 2), 60 s (lane 3), 120 s (lane 4), and 300 s (lane 5) after 100 nM JWH015 stimulation. For quantification of intensity, the density of each band was measured with image analyzing software NIH Image Version 1.63 f, and the ratios of each active form to total Rho-GTPase or PKB/Akt were calculated and normalized to the value of lane 1. Results shown in the bar graphs are the mean values (±S.D.) of four independent experiments (20 experiments total).
consistent with previously reported findings in neutrophil-like HL60 cells (29, 30). These results indicate that the cells had the potential to develop front/rear polarity and that the morphologic alterations we observed constitute a phenomenon peculiar to CB2 stimulation, quite different from alterations induced by other chemoattractants.

As a first step in clarifying the molecular mechanism by which the CB2 ligands induce the curious morphologic alterations, we studied the effects of CB2 stimulation on RhoA, Rac1, Rac2, and Cdc42 activities in neutrophil-like HL60 cells. Activity rates of these four Rho-GTPases over the first 300 s followed different patterns: RhoA activity decreased (Fig. 4A), Rac1 activity increased rapidly at a high level (Fig. 4B), Rac2 activity also increased although more gradually and to a lower peak (Fig. 4C), and Cdc42 activity increased more gradually still (Fig. 4D). One may note that the difference in Rac1 (ubiquitously expressed form of Rac) and Rac2 (predominant form of Rac in neutrophils) activity patterns suggests that these Racs regulate distinct effects and are also differently regulated in neutrophils, as reported by the literature (31, 32).

Our attention, however, was focused on the decrease in RhoA activity because the results were contrary to previous reports concerning RhoA activity following fMLP stimulation in neutrophil-like HL60 cells (29). In addition, we found that pretreatment of human neutrophils with JWH015 or 2-AG substantially suppressed fMLP-induced RhoA activity and also disrupted fMLP-induced front/rear polarization. These results led us to speculate that decreased RhoA activity is involved in the morphologic alterations induced by the CB2 ligands. We then investigated the effects of Rho-dependent protein kinase (p160-ROCK) inhibitor Y27632. We found similarities between the morphologic alterations induced by Y27632 and those induced by JWH015 (Fig. 5, A and B). Xu et al. (29) reported that two inhibitors of RhoA pathways, Y27632 and a dominant-negative RhoA mutant (RhoA-T17), disrupt fMLP-induced cell polarization and induce formation of multiple pseudopods in neutrophil-like HL60 cells. Our results are consistent with theirs, and we believe that the curious morphologic alterations induced by the CB2 ligands are due, at least in part, to suppression of the RhoA-ROCK pathway.

Several studies have shown that myosin II is a major effector of p160-ROCK, which maintains MLC II in its active state (33, 34). Forces generated by activated myosin II-actin complexes are thought to contribute...
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A.

B.

C.

FIGURE 6. Effects of JWH015 and 2-AG on migration in human neutrophils. Human neutrophils were isolated from whole blood and subjected to cell migration assays (A, D, and F) or Rho-GTPase pull-down assays (B, C, and E). A, phase contrast microscopy images of the six groups of neutrophils: unstimulated (panel a), stimulated with 100 nM JWH015 (panel b), 300 nM 2-AG (panel c), or 100 nM fMLP (panel d) and stimulated with fMLP after pretreatment with either JWH015 (panel e) or 2-AG (panel f) for 2 min. Scale bar, 10 μm. B and C, effects of JWH015 or fMLP stimulation on Rac1 (B) and Rac2 (C) in neutrophils. In each of these figures, lanes 1-3 represent Rac activity in unstimulated neutrophils (lane 1), in neutrophils 1 min after stimulation with 100 nM JWH015 (lane 2), or 100 nM fMLP (lane 3). Lanes 4-6 represent the same stimulation but after pretreatment with 1 μM CB2 inhibitor SR144528 for 30 min. The immunoblot images shown are representative of three independent experiments. Upper images show the intensities of total (both GTP- and GDP-bound) Ras. For quantification, the density of each band was measured with image analyzing software NIH Image Version 1.63f, and the ratios of GTP-bound Ras to total Ras were calculated and normalized to the value of lane 1. D, migratory velocities of the nine groups of neutrophils: unpretreated and unstimulated (n = 164), unpretreated and stimulated with 100 nM JWH015 (n = 161), 300 nM 2-AG (n = 175) or 100 nM fMLP (n = 162) for 2 min, stimulated with fMLP after pretreatment with either JWH015 (n = 178) or 2-AG (n = 180) for 2 min, stimulated with fMLP after pretreatment with either JWH015 in the presence of 1 μM CB2 inhibitor SR144528 for 30 min (n = 168), stimulated with fMLP after pretreatment with 2-AG in the presence of either CB2 inhibitor (n = 175) or 1 μM CB1 inhibitor AM251 for 2 min (n = 166). Velocity was calculated from the migratory distance of each cell (excluding non-responding cells) for 15 min (unstimulated cells) or from 5 to 20 min after JWH015, 2-AG, or fMLP stimulation. The bar graph shows the mean velocities (± S.D.) of one typical set of four independent experiments. **, p < 0.01. E, RhoA activity in unstimulated neutrophils (lane 1), in neutrophils stimulated with 100 nM JWH015 (lane 2), 300 nM 2-AG (lane 3), or 100 nM fMLP for 2 min (lane 4) and in neutrophils stimulated with fMLP for 2 min after pretreatment with either JWH015 (lane 5) or 2-AG (lane 6) for 2 min. The immunoblot images shown are representative of three independent experiments. The upper image shows the intensities of GTP-bound (active) RhoA, and the lower image shows the intensities of total (both GTP- and GDP-bound) RhoA. The quantification procedure was as described for B, F, percentage of polarized cells (L/W > 2; see Fig. 2B) in the six groups of human neutrophils described for A. Cells were monitored for 20 min. The bar graph shows the mean values (± S.D.) of four independent experiments.

With regard to the roles of RhoA and Rac in cytoskeletal reorganization and cell polarization, the following have been reported: RhoA plays a role in defining the “rear” and in forming the retracting tail (22, 29, 35); Rac plays a role in defining the “front end” and in forming the lamellipodium containing F-actin (21, 22, 29, 32); RhoA-dependent rear signals inhibit Rac-dependent front end signals and vice versa (29). It is also thought that, in addition to the Rho-GTPases, phosphoinositides are key mediators of cytoskeletal reorganization and that localization of PI3Ps at the front is critical in Rac-mediated F-actin accumulation in lamellipodia (29, 30, 35). Recently, Li et al. (37) showed that at the rear of migrating neutrophils, RhoA-GTP activates p160-ROCK, which in turn activates PI3P phosphatase PTEN (phosphatase and tensin homologue). In the present study, most neutrophil-like HL60 cells that responded to cell polarization (26, 35). Xu et al. (29) reported that exposure to a specific myosin II inhibitor, blebbistatin, induces neutrophil-like HL60 cells to extend multiple pseudopods. Based on these reports, we speculated that the CB2 ligands induced the curious morphologic alterations by suppressing the RhoA-ROCK-MLC II pathway. However, contrary to this speculation, MLC II activity in neutrophil-like HL60 cells increased upon JWH015 stimulation (Fig. 5, D and E). One interpretation of this result is that CB2 stimulation might increase MLC II activity through other pathways that do not involve p160-ROCK. It is reported that the calmodulin-MLC kinase pathway controls lamellipodial extension in leukocytes (36). This pathway may have induced MLCII activation. We confirm that the effects of CB2 ligands on cell morphology do not involve suppression of MLC II activity.
the CB2 ligands developed 1 or more “front” structures (pseudopods containing F-actin), where GFP-PKB/Akt-PH domain (a probe for PI3Ps) accumulated (Fig. 3B, panels b and c), in the absence of a rear structure (a retracting tail). We also found that CB2 stimulation decreased RhoA-GTP (Fig. 4A) but increased Rac-GTP and phosphorylated PKB/Akt (which depends on PI3P level) (Fig. 4, B, C, and E). Our results are consistent with the previously reported findings. In particular, taking our results together with those of Li et al. (37), the following mechanism seems plausible: a decrease in RhoA activity leads to absence of a uropod and a decrease in p160-ROCK activity. In the absence of activated p160-ROCK, PTEN is not activated. If PTEN is not activated, accumulation of PI3Ps is not suppressed in any area of the cell, in turn inducing Rac-mediated F-actin formation in one or more pseudopods. Detailed study is necessary to confirm the involvement of PTEN in CB2 ligand-induced morphologic alterations.

Some previous studies have shown that RhoA activity is connected to structural changes in microtubules (38–41). In particular, Niggli (38) reported that nocodazole-induced microtubule disruption elevates Rho activity. In the present study we observed that CB2 stimulation decreased RhoA activity; thus, direct effects of CB2 on microtubule polymerization are unlikely. However, it is possible that CB2 regulates microtubule structure via Rho-mDia pathway-mediated cytoskeletal effects.

In the present study, we found that in neutrophil-like HL60 cells JWH015 and 2-AG induce motility and morphologic alterations unaccompanied by front/rear polarization and migratory activity. The alterations were induced via suppression of the RhoA-ROCK pathway. In the case of human neutrophils, pretreatment of cells with the CB2 ligands disrupted FMLP-induced front/rear polarization (Fig. 6A, panels e and f) and also substantially suppressed FMLP-induced RhoA activity (Fig. 6F).

Leukocyte polarization is thought to contribute to effective migration to sites of infection and inflammation. Thus, we assessed the influence of the CB2 ligands on neutrophil migratory velocity and found that pretreatment with JWH015 or 2-AG significantly reduced migration induced by FMLP (Fig. 6F). FMLP is a chemotactic peptide produced by bacteria. It is plausible that FMLP is present in vivo when 2-AG is present. It has been shown that lipopolysaccharide- or ionomycin-treated platelets and macrophages produce 2-AG (42). These cells can act in the repair of tissues affected by acute inflammatory cells such as neutrophils.

From these results, we propose that CB2 plays a role in regulating excessive inflammatory response in vivo by controlling RhoA activation, thereby suppressing neutrophil migration.

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