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Cannabinoid Receptor-Mediated Apoptosis Induced by *R*(+)-Methanandamide and Win55,212-2 Is Associated with Ceramide Accumulation and p38 Activation in Mantle Cell Lymphoma[S]

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ABSTRACT

We have recently shown that cannabinoids induce growth inhibition and apoptosis in mantle cell lymphoma (MCL), a malignant B-cell lymphoma that expresses high levels of cannabinoid receptor types 1 and 2 (CB_1 and CB_2). In the current study, the role of each receptor and the signal transduction triggered by receptor ligation were investigated. Induction of apoptosis after treatment with the synthetic agonists *R*(+)-methanandamide [*R*(+)-MA] and Win55,212-2 (Win55; (*R*)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl) pyrrolo-[1,2,3-*d*,*e*]-1,4-benzoxazin-6-yl]-1-naphthalenyl-methanone) was dependent on both cannabinoid receptors, because pretreatment with *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboximide hydrochloride (SR141716A) and *N*-((1*S*)-endo-1,3,3-trimethyl bicyclo heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide) (SR144528), specific antagonists to CB_1 and CB_2, respectively, abrogated caspase-3 activity. Preincubation with the inhibitors 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole (SB203580) and 4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)-1*H*-imidazole (SB202190) showed that phosphorylation of MAPK p38 was implicated in the signal transduction leading to apoptosis. Treatment with *R*(+)-MA and Win55 was associated with accumulation of ceramide, and pharmacological inhibition of ceramide synthesis de novo prevented both p38 activation and mitochondria depolarization assessed by binding of 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$). In contrast, the pan-caspase inhibitor z-Val-Ala-Asp(Ome)-CH_2F (z-VAD-FMK) did not protect the mitochondrial integrity. Taken together, these results suggest that concurrent ligation of CB_1 and CB_2 with either *R*(+)-MA or Win55 induces apoptosis via a sequence of events in MCL cells: accumulation of ceramide, phosphorylation of p38, depolarization of the mitochondrial membrane, and caspase activation. Although induction of apoptosis was observed in both MCL cell lines and primary MCL, normal B cells remained unaffected. The present data suggest that targeting CB_1/ CB_2 may have therapeutic potential for the treatment of mantle cell lymphoma.

MCL is a malignant B-cell lymphoma with an aggressive course and generally a poor clinical outcome. MCL tumors respond to chemotherapy, but the remissions are short and the median survival is only 3 years. Using gene expression profiling to identify potential new therapeutic targets in MCL, we recently reported overexpression of the $G_{i/o}$-protein-coupled receptors CB_1 and CB_2 (Islam et al., 2003). Under normal conditions, the CB_1 receptor is predominantly expressed in the central nervous system, whereas CB_2 is mainly expressed in the immune system. In addition to their roles in neuromodulation and immune function, the canna-

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ABBREVIATIONS: MCL, mantle cell lymphoma; CB_1 and CB_2, cannabinoid receptors type 1 and 2; MAPK, mitogen-activated protein kinase; *R*(+)-MA, *R*(+)-methanandamide; Win55, Win55,212-2 [(*R*)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl) pyrrolo-[1,2,3-*d*,*e*]-1,4-benzoxazin-6-yl]-1-naphthalenyl-methanone]; SR141716A, *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); SB202190, 4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)-1*H*-imidazole; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole; PBS, phosphate-buffered saline; PE, phycoerythrin; FCS, fetal calf serum; B-PLL, B-prolymphocytic leukemia; 7AAD, 7-amino-actinomycin D; MS/MS, tandem mass spectrometry; IS, internal standard; SPL, sphingolipid; dh, dihydro; $DiOC_6$, 3,3′-dihexyloxacarbocyanine iodide; z-VAD-FMK, *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone; FB1, Fumonisin B1; L-CS, L-cycloserine.

binoid receptors participate in the regulation of cell survival (Guzman et al., 2001b). Endogenous as well as natural and synthetic cannabinoids act as agonists to the receptors (Howlett et al., 2002) and have been shown to induce growth inhibition in vitro and tumor regression in vivo in a variety of cell types (Guzman, 2003). Both cell-cycle arrest and apoptosis have been reported to contribute to cannabinoid receptor-mediated growth inhibition (Guzman, 2003). The cellular signal transduction induced by cannabinoids is complex and depends both on the cellular context and on the kind and duration of the stimulus. Cannabinoids can modulate mitogen-activated protein kinases (MAPKs) that are generally believed to play opposing roles in the control of cell growth (Wada and Penninger, 2004). Both cannabinoid-induced attenuation (Ellert-Miklaszewska et al., 2005) and activation (Galve-Roperh et al., 2000) of ERK signaling, which is normally associated with cell survival, have been reported to mediate apoptosis via inhibition of the AKT/protein kinase B survival pathway (Gomez del Pulgar et al., 2002; Ellert-Miklaszewska et al., 2005). Activation of the stress-activated kinases c-Jun NH_2-terminal kinase and p38 MAPK in response to cannabinoid receptor ligation has also been reported (Liu et al., 2000; Rueda et al., 2000; Derkinderen et al., 2001). In some cases, cannabinoids have shown to induce accumulation of ceramide (Derkinderen et al., 2001; Velasco et al., 2005), a sphingolipid that acts as a second messenger (Pettus et al., 2002). Increased ceramide levels in response to treatment with cannabinoids can be caused by either sphingomyelin breakdown (Sanchez et al., 1998) or by de novo ceramide synthesis (Galve-Roperh et al., 2000; Gomez del Pulgar et al., 2002). The de novo pathway is activated in response to TNF and several chemotherapeutic agents and has been suggested to play a critical role in the regulation of apoptosis (Pettus et al., 2002). In glioma (Galve-Roperh et al., 2000; Gomez del Pulgar et al., 2002) and prostate tumor cells (Mimeault et al., 2003) treated with cannabinoids, de novo ceramide accumulation has been shown to precede apoptosis.

We have observed previously that treatment with cannabinoid receptor agonists leads to growth inhibition and induction of apoptosis in both primary MCL cells and in the MCL cell line Rec-1 (Flygare et al., 2005). The objective of the present investigation was to clarify the role of each receptor and to delineate the signaling pathway leading to apoptosis in MCL after treatment with the endocannabinoid analog *R*(+)-MA and the synthetic cannabinoid Win55. In MCL cell-lines and primary MCL cells, induction of apoptosis after treatment with *R*(+)-MA and Win55 was dependent on concurrent ligation of CB_1 and CB_2, whereas normal B cells remained unaffected. A sequential connection between signaling events was mapped: cannabinoid receptor ligation was followed by ceramide synthesis before p38 activation, which preceded a drop in the mitochondrial membrane potential and, finally, caspase activation. Given the low expression of CB_1 in normal immune cells, our results suggest that cannabinoid receptor ligands could be developed as novel therapeutic agents for the treatment of MCL.

Materials and Methods

Ethical Permission. This study was approved by the Ethics committee at Karolinska Institutet (Etikkommitté syd).

Reagents. SR141716A and SR144528 were kind gifts from Sanofi-Aventis (Montpellier, France). *R*(+)-Methanandamide was purchased from Tocris Cookson Inc. (Bristol, UK). Win 55,212-2 mesylate, Fumonisin B1, L-CS, and z-VAD-FMK were purchased from Sigma Aldrich (St Louis, MO). SB202190 and SB203580 were purchased from Calbiochem Inc. (San Diego, CA), and 3,3′-dihexyloxacarbocyanine iodide was purchased from Invitrogen (Carlsbad, CA). *R*(+)-MA was dissolved to 10 mM in EtOH and the other reagents were dissolved to 10 mM in dimethyl sulfoxide. Further dilutions in culture medium were made on the day of the experiment.

Tumor Tissue and Controls. Tumor tissue from MCL was obtained from cryopreserved patient samples with more than 90% purity of tumor B cells. Three samples (L718, L1547, and L1676) were collected from tonsil, lymph node, and peripheral blood, respectively, of three MCL patients. In all instances, the MCL diagnosis was based on typical morphology, immune phenotype, expression of cyclin D1 protein and/ or t(11;14)(q13;q32) assessed by fluorescence in situ hybridization. Control samples consisted of B lymphocytes isolated from peripheral blood or from nonmalignant tonsil tissue. MCL and tonsil tissue were homogenized using Medimachine (Dako Denmark A/S, Glostrup, Denmark), resuspended, and washed in PBS.

B Lymphocyte Separation. Human peripheral blood lymphocytes were separated from blood cell concentrates of healthy blood donors by density centrifugation on Ficoll-Paque gradient (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Erythrocytes were lysed in ammonium chloride lysis buffer for 8 min. The cells were resuspended in RPMI 1640 medium and stored at 4°C overnight. Human B lymphocytes from tonsil tissue and blood were separated from T cells by AutoMACS, using 160 μl of CD3+ microbeads to 8.7×10^6 cells. The B cells separated from buffy coat were stained with PE-Cy5 anti-human CD19 (BD Biosciences PharMingen, San Jose, CA) for 15 min at RT, in the dark. Cells were washed and sorted for CD19+ in FACSVantage SE/DiVa sorter (BD Biosciences) with an excitation wavelength of 488 nm.

Cell Lines. The MCL cell line Rec-1, carrying the t(11;14)(q13;q32) translocation (Rimokh et al., 1994) was a kind gift from Dr. Christian Bastard (Ronan, France). Rec-1 and the myelomonocytic cell line U937 cells were maintained in RPMI 1640 medium supplemented with 2 mM L-glutamine and 10% fetal calf serum (FCS) and 50 μg/ml gentamicin under standard conditions (humidified atmosphere, 95% air, 5% CO_2, 37°C). The Epstein-Barr virus-negative MCL cell line JeKo (Jeon et al., 1998) and the JVM-2 cell line generated from a B-prolymphocytic leukemia (B-PLL) with t(11;14)(q13;q32) (Melo et al., 1986) were kept under the same conditions except for supplementation with 20% FCS. Several studies have suggested that B-PLL is not a specific disease entity, but a blastic variant of a different lymphoma. Most cases carrying the t(11;14) translocation correspond to blastoid variants of MCL (Ruchlemer et al., 2004). Before performing the experiments, cells were serum-starved overnight.

Apoptosis and Flow Cytometric Analysis. Cells were serum-starved overnight, treated with 10 μM Win55 or *R*(+)-MA, and harvested 4 h after the treatment. The cells were labeled with Annexin V-PE and 7AAD using the Annexin V-PE Apoptosis Detection kit (BD Biosciences Pharmingen) according to the manufacturer's instructions. Subsequent flow cytometric analysis was performed on a FACScan (BD Biosciences). Fluorescence data were displayed as dot plots using the CellQuest software (BD Biosciences).

Caspase-3 Assay. DEVD-dependent caspase activity was measured using a Caspase-3/CPP32 Fluorometric Assay (MCL International, Woburn, MA) according to the manufacturer's instructions. The assay is based on fluorometric detection of cleavage of the substrate DEVD-AFC. Uncleaved substrate emits blue light (λ_{max}, 400 nm), whereas free 7-amino-4-trifluoromethyl coumarin emits yellow-green light fluorescence (λ_{max}, 505 nm). In brief, cells from cell lines were serum-starved overnight, whereas primary MCL and tonsil tissue cells were used directly after preparation. Cells were

pretreated with inhibitors for 30 min or 1 h (see figure legends) before incubation with 10 μM $R(+)$-MA or Win55 for 30 min. Cells were then harvested, lysed, and incubated with buffer containing DEVD-7-amino-4-trifluoromethyl coumarin for 2 h at 37°C. Emission was measured using a fluorometer at excitation and emission wavelengths of 400 nm and 505 nm, respectively.

Enzyme Immunometric Phosphorylation Assays. The amount of phosphorylated p38 and total p38 in the cells was measured using pp38 and p38 enzyme immunoassays (Assay Designs Inc., Ann Arbor, MI) according to the manufacturer's instructions. In brief, Rec-1 cells were serum-starved overnight, pretreated with inhibitors for 30 min or 1 h (see figure legends) before a 30-min incubation with 10 μM $R(+)$-MA or Win55. Thereafter, the cells were washed twice with PBS and lysed in radioimmunoprecipitation assay buffer (Assay Designs Inc.) containing protease inhibitor cocktail p8340 (Sigma-Aldrich). The cell lysate was allowed to bind to the enzyme immunoassay plate for 1 h. The microtiter plate was incubated with phosphospecific antibodies against phosphorylated p38 or against total p38 for 1 h on a plate shaker, followed by washing and incubation with secondary conjugate solution for 30 min. Thereafter, TMB substrate was added for 30 min. Adding stop solution terminated the reaction, and the emission at 450 nm was determined. The ratio of phosphorylated/total p38 MAPK was then calculated, and results were expressed as a percentage of the ratio calculated for unstimulated control cells.

Ceramide Analysis. High-performance liquid chromatography-tandem mass spectrometry (MS/MS) was performed at the Lipidomics Core, Medical University of South Carolina (Charleston, SC) as described by Bielawski et al. (2006). In brief, Rec-1 cells were serum-starved overnight, whereas B lymphocytes from tonsil tissue were used directly after preparation. The cells were incubated with 10 μM $R(+)$-MA or Win55 for 4 h. Thereafter, simultaneous electrospray ionization (ESI) MS/MS analysis of sphingoid bases, sphingoid base 1-phosphates and ceramides was performed on a triple quadrupole mass spectrometer (TSQ 7000; Thermo Electron Corporation, Waltham, MA) operating in a multiple reaction-monitoring positive ionization mode. Cells were fortified with internal standards (IS), extracted into a one-phase neutral organic solvent system, and analyzed by a HP 1100/TSQ 7000 liquid chromatography/MS system. Qualitative analysis of sphingolipids (SPLs) was performed by a parent ion scan of a common fragment ion characteristic for a particular class of SPLs. Quantitative analysis was based on calibration curves generated by spiking an artificial matrix with known amounts of target synthetic standards and an equal amount of IS. The calibration curves were constructed by plotting the peak area ratios of analyte to the respective IS against concentration using a linear regression model. SPL levels were normalized to total lipid phosphate.

Mitochondrial Transmembrane Potential. 3,3′-Dihexyloxacarbocyanine iodide ($DiOC_6$) is a strong cationic dye that binds to undamaged mitochondria with intact membrane potential. To evaluate changes in the mitochondrial transmembrane potential, Rec-1 cells were serum-starved overnight and pretreated with inhibitors for 30 min or 1 h (see figure legends) before incubation with 10 μM $R(+)$-MA or Win55 for 30 min. After washing and resuspension in RPMI 1640 medium without FCS and phenol red, cells were labeled

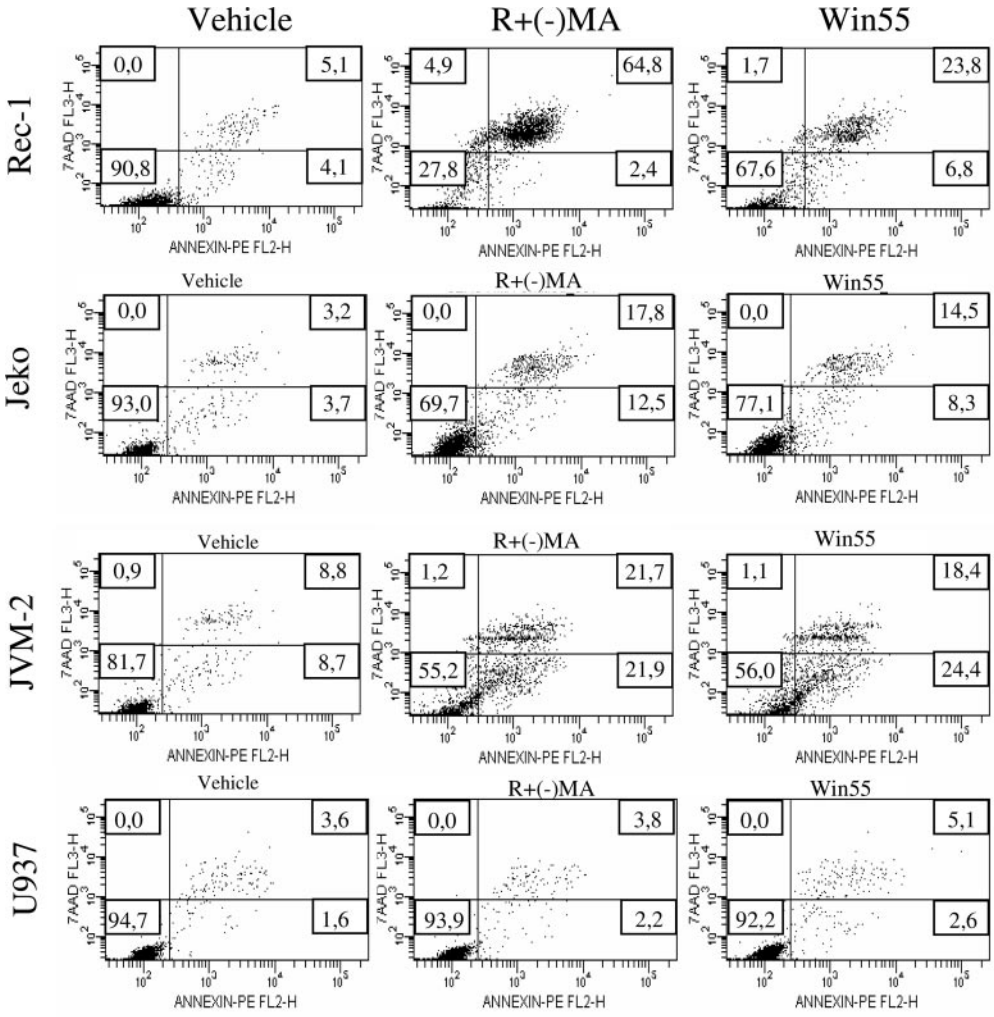

Fig. 1. Cannabinoids induce cell death in MCL cell lines. The MCL cell lines Rec-1, JeKo, and JVM-2 and the control cell line U937 were serum-starved overnight and treated with vehicle or 10 μM $R(+)$-MA or Win55. Flow cytometry analysis of AnnexinV-PE and 7AAD was performed 4 h after treatment. Top and bottom right quadrants show late apoptotic/necrotic and early apoptotic cells, respectively. Representative results from three individual experiments are shown.

with $DiOC_6$ (40 nM) for 15 min at 37°C. Thereafter, cells were washed twice with PBS and analyzed directly on a flow cytometer using excitation and emission wavelengths of 495 and 525 nm, respectively.

Statistical Analysis. Caspase-3 activity and p38 phosphorylation were evaluated using Kruskal-Wallis test between control cells and treated cells. *P* values are presented in figure legends. The software Statistica (Statsoft AB, Tulsa, OK) was used.

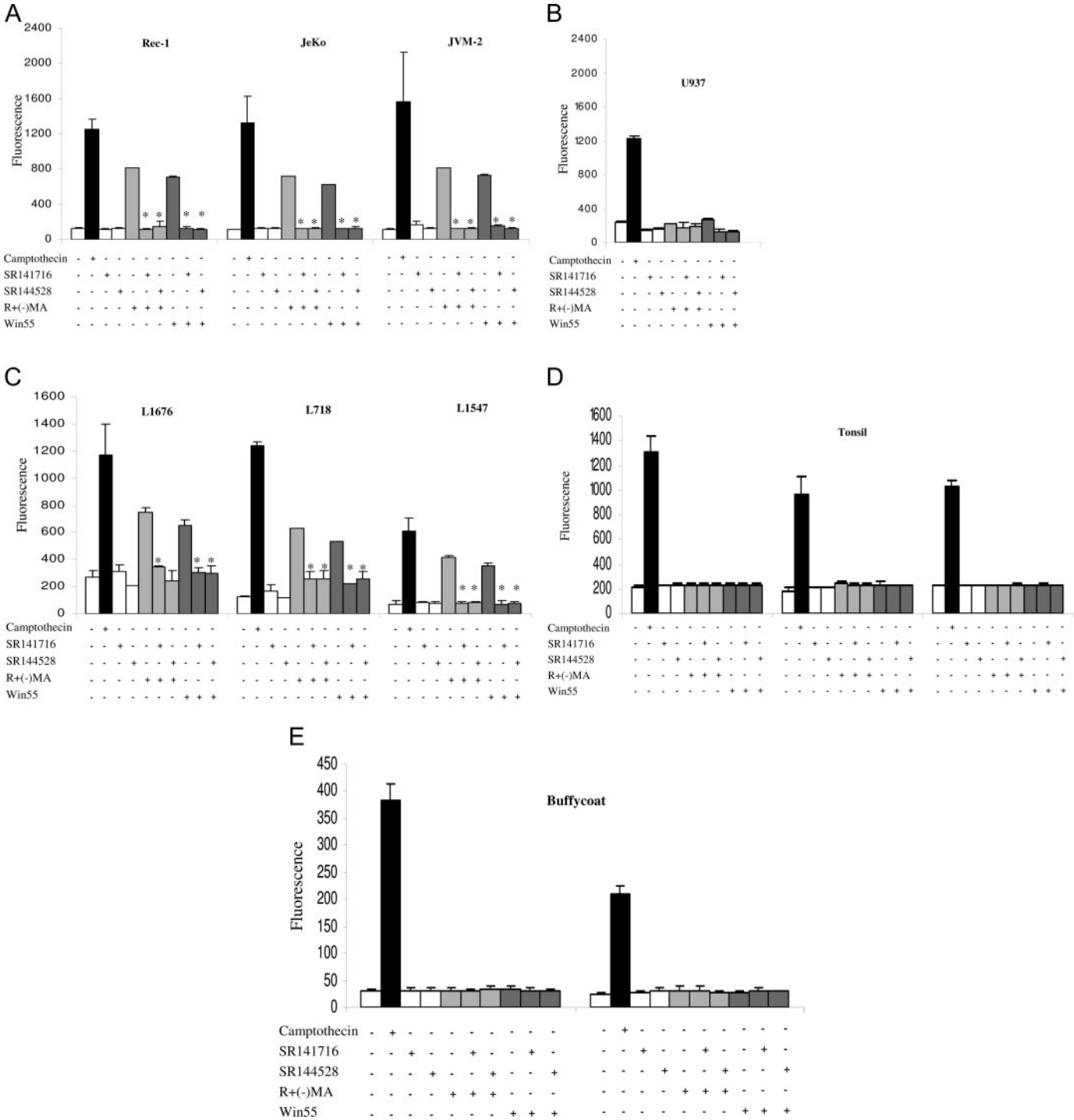

Fig. 2. Cannabinoids induce caspase-3 activity in MCL but not in normal B cells. The caspase-3 fluorometric assay was performed, and fluorescence units are shown on the *y*-axis. One of two individually performed experiments is shown. *, $p < 0.05$ when agonist treated cells with and without inhibitors were compared, Kruskal-Wallis analysis. Role of CB_1 and CB_2 in cannabinoid-induced cell death in MCL cell lines. Rec-1, JeKo, and JVM-2 (A) or control U937 (B) cells were serum-starved overnight and pretreated with the CB_1 antagonist SR141716A (10 nM) or the CB_2 antagonist SR144528 (10 nM) for 30 min before incubation with vehicle, 10 μM *R*(+)-MA (light gray) or Win55 (dark gray) for 30 min. Camptothecin (50 μM; black) was used as positive control. Role of CB_1 and CB_2 in cannabinoid-induced cell death in primary MCL cells. C, primary MCL from three cryopreserved patient samples and primary B lymphocytes from (D) tonsil tissue from three patients or (E) buffy coat from two healthy blood donors were pretreated with the CB_1 antagonist SR141716A (10 nM) or the CB_2 antagonist SR144528 (10 nM) for 30 min before incubation with vehicle, 10 μM *R*(+)-MA (light gray), or Win55 (dark gray) for 30 min; 50 μM camptothecin (black) was used as positive control.

Results

Induction of Apoptosis by *R*(+)-MA and Win55. First, we examined whether exposure of the MCL cell lines Rec-1, JeKo, and JVM-2 to *R*(+)-MA, which displays higher affinity for CB_1 than for CB_2 (Howlett et al., 2002) or Win55, which binds to CB_1 and CB_2 with similar affinities (Howlett et al., 2002), would lead to induction of apoptosis. Cell death was detected by flow cytometry analysis of cells labeled with Annexin-PE and 7AAD. *R*(+)-MA or Win55 (10 μM) caused apoptosis and necrosis to various extents in the different MCL cell lines, whereas the control cell line U937, which does not express CB_1 or CB_2 (Maccarrone et al., 2000), remained unaffected (Fig. 1).

To assess the individual contributions of the CB1 and CB2 receptors to the apoptotic response to treatment with *R*(+)-MA and Win55, studies with inhibitors were performed. Cells were pretreated with SR141716A or SR144528, specific inhibitors of CB1 and CB2, respectively, followed by exposure to 10 μM *R*(+)-MA or Win55. Caspase-3 activity was measured using a quantitative assay. SR141716A or SR144528 (10 nM) abrogated the caspase-3 activity observed in the MCL cell lines (Fig. 2A) and in primary MCL samples (Fig. 2C) treated without inhibitor. Thus, ligation of both CB1 and CB2 seems to be required for the induction of apoptosis in MCL cells. In contrast, the control cell line U937 (Fig. 2B) and normal B lymphocytes from tonsil tissue (Fig. 2D) or buffycoat (Fig. 2E) were only marginally affected by treatment with the agonists.

Cannabinoid Receptors Mediate p38 Phosphorylation Followed by Caspase-3 Activation. MAPK signaling has earlier been shown to mediate apoptosis induced by cannabinoids in different cell types (Howlett and Mukhopadhyay, 2000; Wada and Penninger, 2004; Ellert-Miklaszewska et al., 2005). Therefore, the potential role of MAPK signaling in the induction of apoptosis in the MCL cell line Rec-1 was investigated. Pretreatment of Rec-1 with 10 μM concentrations of the p38 inhibitors SB202190 or SB203580 before incubation with 10 μM *R*(+)-MA or Win55 caused a substantial decrease in caspase-3 activity compared with the samples treated without inhibitors (Fig. 3A). In contrast, the c-Jun NH_2-terminal kinase inhibitor SP500125 and the p44/42 inhibitor UO126 were less effective (data not shown).

Activation of p38 in response to treatment with *R*(+)-MA or Win55 was confirmed by enzyme-linked immunosorbent assays specific for phospho p38 and total p38. Pretreatment with 10 nM SR141716A or SR144528 for 30 min before incubation with 10 μM *R*(+)-MA or 10 μM Win55 caused an almost complete inhibition of the p38 phosphorylation observed in the samples treated without inhibitors (Fig. 3B). Thus, p38 phosphorylation and caspase-3 activation are mediated in a similar way by ligation of both the CB_1 and the CB_2 receptor.

Ceramide Accumulation Precedes p38 Activation. Recent studies have suggested that apoptosis induced by cannabinoids can be preceded by ceramide accumulation (Derkinderen et al., 2001). In view of these observations, ceramide formation in response to treatment with *R*(+)-MA and Win55 was studied in Rec-1 and normal B lymphocytes from tonsil. To measure levels of different ceramide subspecies ranging from C12 to C26 in fatty acid length, high-performance liquid chromatography–tandem mass spectrometry was employed. In Rec-1, 10 μM cannabinoid receptor agonists induced 2.3- [*R*(+)-MA] and 1.5-fold (Win55) increases in both C14 (Fig. 4A) and C16 (Fig. 4B). During de novo ceramide synthesis, dihydroceramide species serve as

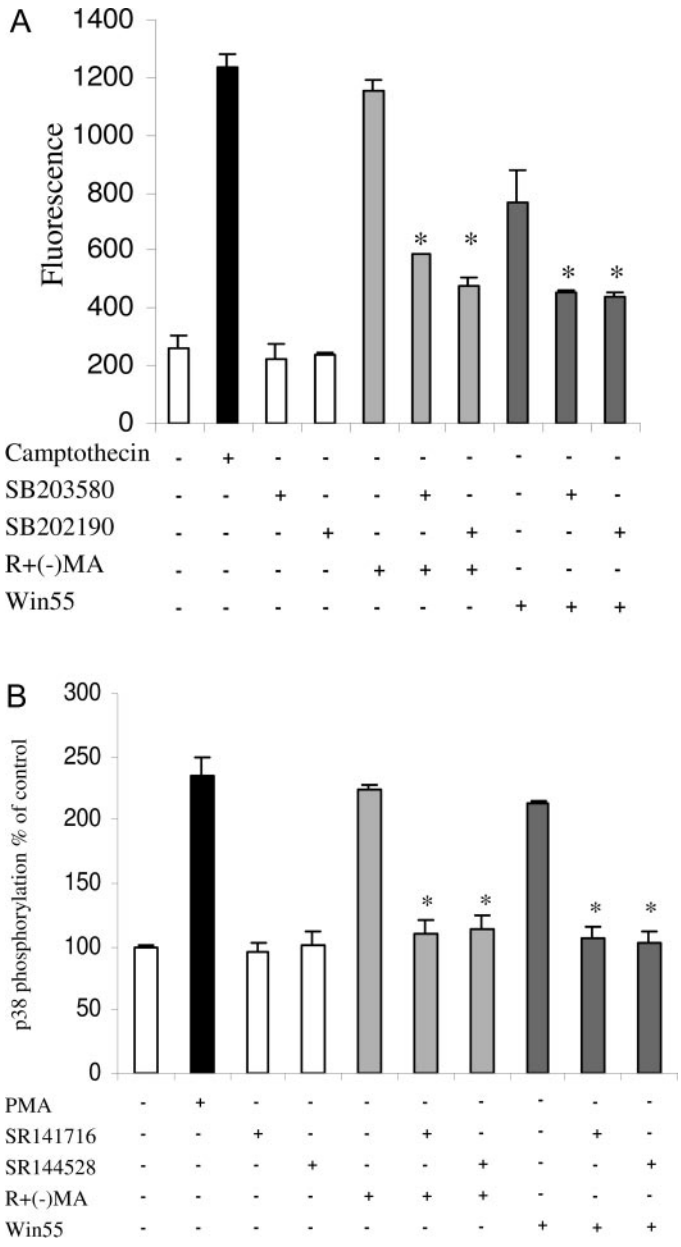

Fig. 3. Cannabinoid-induced p38 phosphorylation. One of two individually performed experiments is shown. *, $p < 0.05$ when agonist treated cells with and without inhibitors were compared, Kruskal-Wallis analysis. A, role of p38 in caspase-3 activation. Rec-1 cells were serum starved overnight and pretreated with the p38 inhibitors SB203580 (10 μM) or SB202190 (10 μM) for 30 min before incubation with vehicle, 10 μM *R*(+)-MA (light gray) or Win55 (dark gray) for 30 min; 50 μM camptothecin (black) was used as positive control. The caspase-3 fluorometric assay was performed, and fluorescence units are shown on the *y*-axis. *B,* contribution of CB_1 and CB_2 to p38 activation. Rec-1 cells were serum-starved and pretreated with the CB_1 antagonist SR141716A (10 nM) or the CB_2 antagonist SR144528 (10 nM) for 30 min before treatment with 10 μM *R*(+)-MA (light gray) or Win55 (dark gray) for 30 min. PMA (3 μM; black) was used as a positive control. To assess p38 MAPK activity, both phosphorylated and total p38 MAPK immunoassays were performed. The ratio of phosphorylated/total p38 MAPK was then calculated and results are expressed as a percentage of the ratio calculated for unstimulated control cells.

precursors to ceramide species (Pettus et al., 2002). dhC14 and dhC16, the dihydroceramide counterparts of C14 and C16, both increased 5-fold after treatment with *R*(+)-MA and >3-fold after treatment with Win55 in Rec-1 (Fig. 4, C and D). Increased levels of dhC18, dhC20, dhC24, dhC26:1, and dhSphingosine were also observed in Rec-1 (see Supplementary Information). In general, the induction of ceramide and dihydroceramide was less pronounced in B lymphocytes from tonsil compared with Rec-1 (Fig. 4, A–D, and Supplementary Information). Increased levels of dihydroceramide in response to the cannabinoid receptor agonists in MCL suggest that the de novo ceramide synthesis pathway was activated.

To determine whether ceramide accumulation occurs upstream or downstream of p38 activation, we next examined the effects of pretreatment with inhibitors of de novo ceramide synthesis on p38 activation. Fumonisin B1 (FB1) and L-CS, inhibitors of dihydroceramide synthase and of ketosphinganine synthase, respectively, were used. As shown in Fig. 5, a 25 μM concentration of inhibitors prevented p38 phosphorylation induced by 10 μM *R*(+)-MA or Win55 in Rec-1. Thus, de novo ceramide accumulation induced by *R*(+)-MA or Win55 is followed by p38 phosphorylation in MCL.

Ceramide Accumulation Causes Changes in the Mitochondrial Membrane Potential Upstream of Caspase Activation. Ceramide accumulation is associated with mitochondrial loss of function, release of cytochrome *c*, and caspase activation (Pettus et al., 2002). Mitochondrial damage can be assessed biochemically by measuring $DiOC_6$, a cationic dye that is released when the mitochondrial membrane potential is damaged. A clear decrease in the binding of $DiOC_6$ was observed in Rec-1 cells treated with 10 μM Win55 or *R*(+)-MA (Fig. 6, column 1). Pretreatment with 10 nM SR141716A or SR144528 (Fig. 6, column 2 and 3) protected the mitochondrial integrity. Moreover, the drop in $DiOC_6$ binding was inhibited by FB1 (Fig. 6, column 4) or SB202190 (Fig. 6, column 5), suggesting that ceramide accumulation

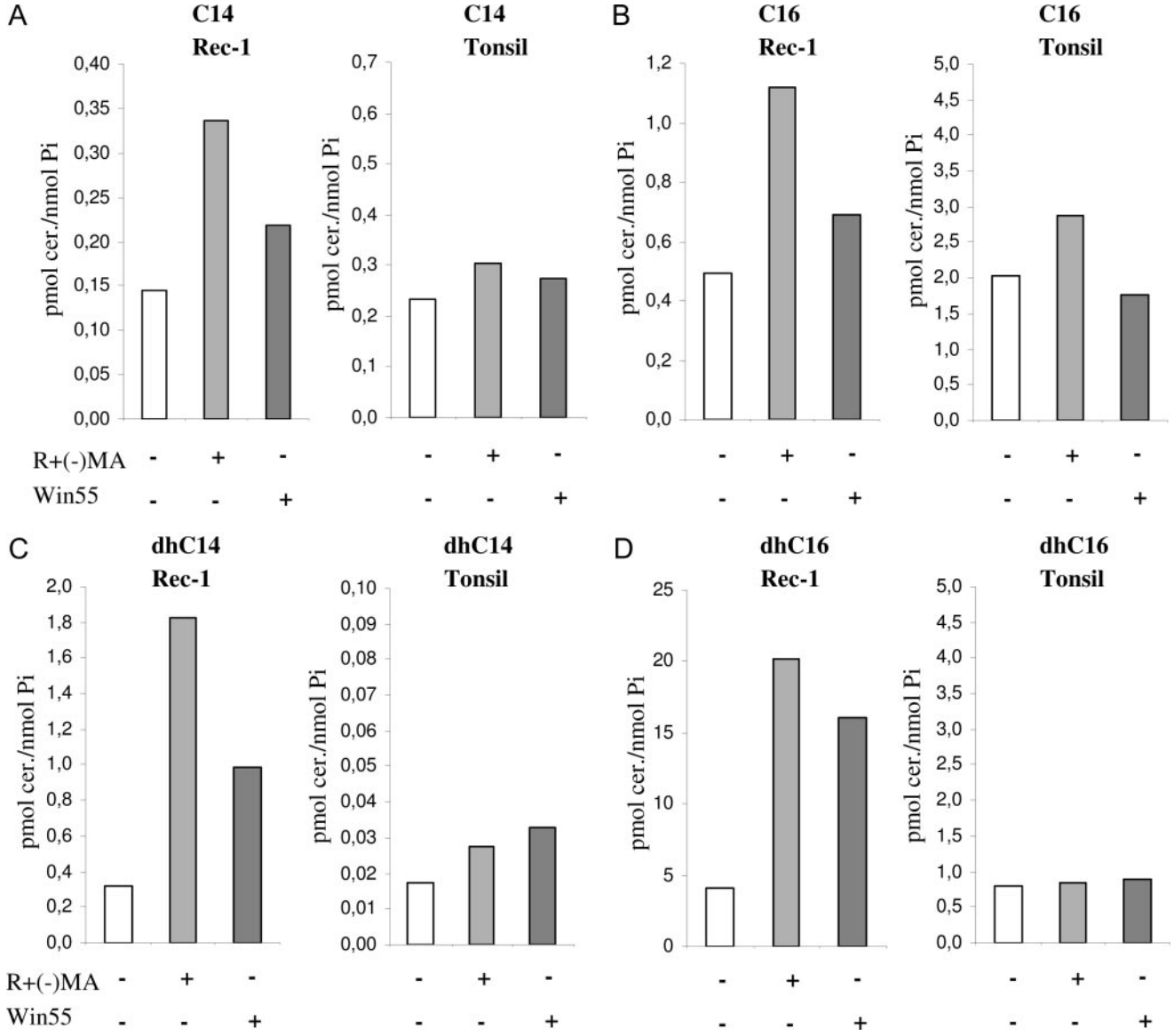

Fig. 4. Cannabinoid-induced accumulation of ceramide and dihydroceramide. Rec-1 (serum starved overnight) and B lymphocytes from tonsil were treated with vehicle, 10 μM *R*(+)-MA (light gray) or Win55 (dark gray) for 4 h. Thereafter, the cells were prepared for measurement of ceramide using high-performance liquid chromatography-tandem mass spectrometry as described. The masses of the ceramide subspecies C14 (A) and C16 (B) and their corresponding dihydroceramides dhC14 (C) and dhC16 (D) were normalized to lipid phosphate (P_i).

and p38 activation precede loss of mitochondrial membrane potential. In contrast, the pan-caspase inhibitor z-VAD-FMK did not protect against mitochondrial loss of function (Fig. 6, column 6), indicating that caspases are activated downstream of mitochondrial depolarization in MCL cells.

Discussion

Various kinds of cannabinoids have been shown to induce apoptosis in human leukemia and lymphoma cell lines via CB_2, the cannabinoid receptor normally expressed in the immune system (McKallip et al., 2002a). We have demonstrated recently that cannabinoid receptor ligands induce growth reduction and apoptosis in MCL (Flygare et al., 2005), a B-cell lymphoma that expresses high levels of both CB_1 and CB_2 (Islam et al., 2003). In the current study, we examined the role of each receptor, and investigated involvement of MAPK signaling and ceramide accumulation in the induction of apoptosis by two synthetic cannabinoids. Together, our data suggest that targeting CB_1 and CB_2 receptors may constitute a novel approach to treating MCL.

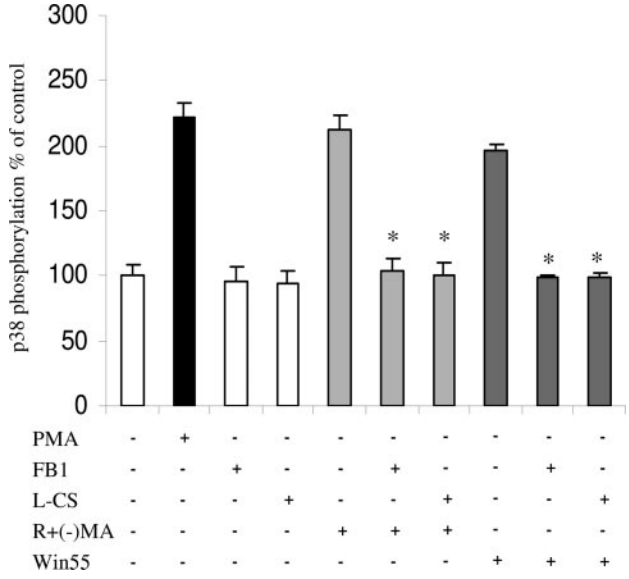

Fig. 5. Cannabinoid-induced ceramide accumulation is required for p38 phosphorylation. Rec-1 cells were serum-starved overnight and pretreated with 25 μM FB1 and L-CS for 1 h before treatment with vehicle, 10 μM $R(+)$-MA (light gray) or Win55 (dark gray) for 30 min. PMA (3 μM; black) was used as a positive control. To assess p38 MAPK activity, both phosphorylated and total p38 MAPK immunoassays were performed. The ratio of phosphorylated/total p38 MAPK was then calculated and results are expressed as a percentage of the ratio calculated for unstimulated control cells. One of two individually performed experiments is shown. *, $p < 0.05$ when agonist-treated cells with and without inhibitors were compared, Kruskal-Wallis analysis.

First, we showed that both $R(+)$-MA and Win55 induced apoptosis in MCL. The effect was more pronounced after treatment with the CB_1 selective agonist $R(+)$-MA compared with the mixed CB_1/CB_2 agonist Win55. Programmed cell death in response to Win55 treatment has previously been observed in skin cancer (Casanova et al., 2003) and prostate cancer cells (Sarfaraz et al., 2005) that express both CB_1 and CB_2. $R(+)$-MA has recently been shown to induce apoptosis in a cannabinoid receptor independent manner in glioma cells (Hinz et al., 2004). In MCL, caspase-3 activation induced by either $R(+)$-MA or Win55 was completely reversed by preincubation with 10 nM SR141716A or SR144528. At this dose, the inhibitors act merely as neutral antagonists to CB_1 and CB_2, respectively, whereas roles as inverse agonists have been suggested at higher doses (Pertwee, 2005). Therefore, our current data suggest that $R(+)$-MA and Win55, irrespective of differences in CB_1/CB_2 selectivity, induce caspase-3 mediated apoptosis in MCL cells via ligation of both cannabinoid receptors. A similar requirement of ligation of both CB_1 and CB_2 has been observed after treatment of dendritic cells with Δ^9-tetrahydrocannabinol (Do et al., 2004).

The major part of the studies on growth inhibition in response to cannabinoids have been performed in neuronal cells, where modulation of ERK and AKT/protein kinase B have been implicated in the signaling leading to apoptosis (Guzman, 2003). In contrast, we found that caspase-3 activity in MCL cells was to a large extent dependent on phosphory-

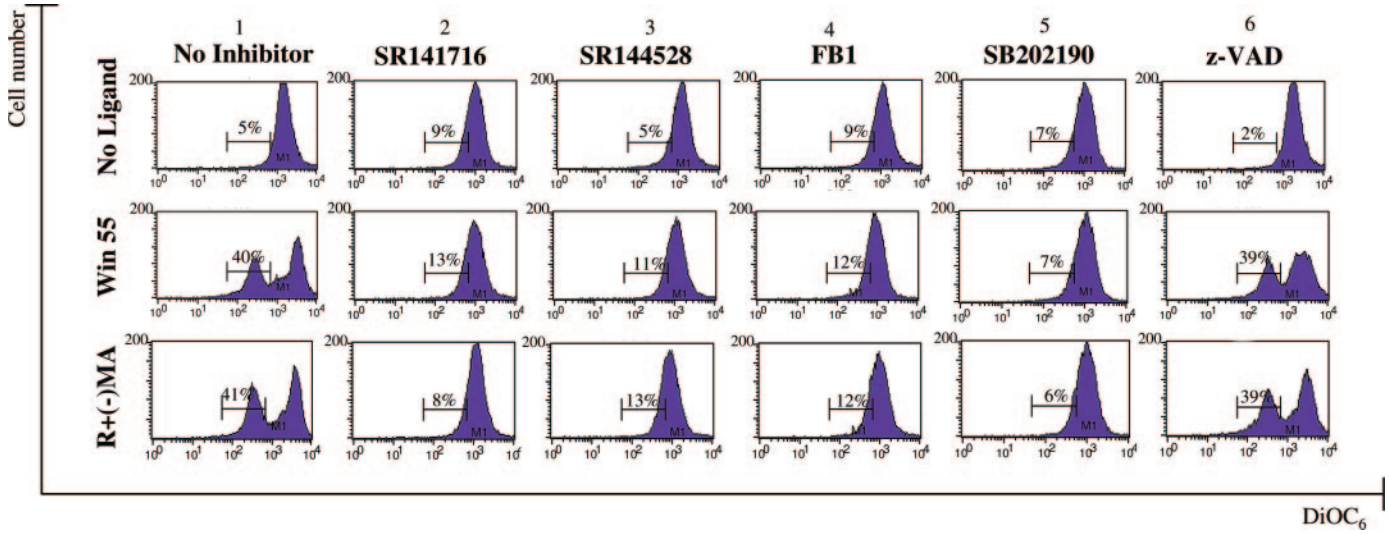

Fig. 6. Cannabinoids induce changes in mitochondrial membrane potential downstream of ceramide accumulation and p38 activation but upstream of caspase activation. Rec-1 cells were serum-starved overnight and pretreated with SR141716A (10 nM), SR144528 (10 nM), FB1 (25 μM), SB202190 (10 μM), or z-VAD-FMK (50 μM) for 30 min before incubation with 10 μM $R(+)$-MA or Win55 for 30 min. Thereafter, flow cytometric analysis of mitochondrial membrane potential was performed using staining with $DiOC_6$. Representative results from three individual experiments are shown.

lation of p38, a MAPK whose activation can promote either apoptosis or survival depending on cell type (Wada and Penninger, 2004). Activation of this kinase before apoptosis induced by IgM cross-linking has earlier been reported in B cells from patients with B-chronic lymphocytic leukemia (Nedellec et al., 2005).

Recent studies have shown that the CB_1 receptor is coupled to the generation of ceramide, reviewed in Guzman et al. (2001a). *R*(+)-MA and Win55 have been shown to induce ceramide accumulation in neuronal cells (Galve-Roperh et al., 2000; Ramer et al., 2003). Our results have shown that either agent induced accumulation of C14 and C16 ceramide and their respective dihydroceramide counterparts in Rec-1. In accordance with the effects of the agonists on induction of apoptosis (Fig. 1 upper row), *R*(+)-MA was more potent than Win55. The magnitude of the changes in ceramide content was similar to that observed during B cell receptor triggered cell death in Ramos cells (Kroesen et el., 2001). Induction of dihydroceramide synthesis suggests that the de novo ceramide synthesis pathway was activated. Moreover, pharmacological inhibition of de novo ceramide synthesis inhibited phosphorylation of p38 and changes in mitochondrial membrane potential. In contrast to ceramides C14 and C16, no induction of longer chain ceramides was observed. However, increased levels of longer chain dihydroceramides (dhC18-dhC26:1) indicate that changes in the corresponding ceramides may be observed at later time points.

Ceramide formation has been reported to induce both caspase-dependent and -independent apoptosis (Zhao et al., 2004) and can either precede caspase activation or occur in between initiator caspases and effector caspases (Tepper et al., 1999; Kroesen et al., 2001). The pan-caspase inhibitor z-VAD-FMK did not affect the drop in mitochondrial membrane potential caused by *R*(+)-MA and Win55 in Rec-1. Thus, ceramide accumulation precedes loss in mitochondrial membrane potential, which is placed upstream of caspase activation in MCL cells. In accordance with earlier studies in neuronal cells (Stoica et al., 2005), p38 inhibition blocked cannabinoid signaling upstream of the mitochondria in MCL. p38 may function both upstream and downstream of caspases, as reviewed in Zarubin and Han (2005). Taken together, the present observations suggest a signaling model in which concurrent ligation of CB_1 and CB_2 mediates cannabinoid-induced apoptosis via accumulation of ceramide, phosphorylation of p38, depolarization of the mitochondrial membrane, and caspase activation (Fig. 7).

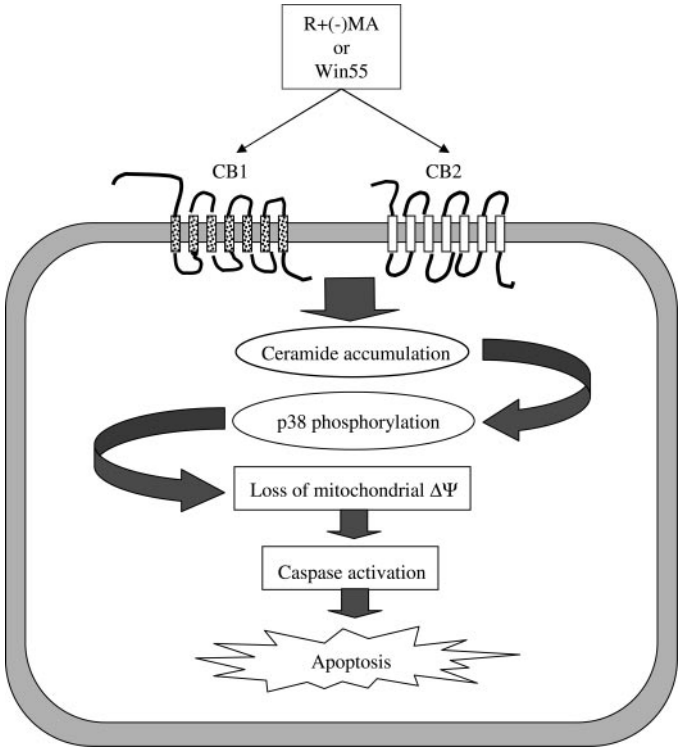

Fig. 7. Proposed model of signaling events induced by the cannabinoid receptor agonists *R*(+)-MA or Win55 in MCL. ΔΨ, membrane potential.

Cannabinoids have been shown to selectively suppress the growth of glioma, thyroid epithelioma, and skin carcinoma cells compared with their normal counterparts (Guzman, 2003), which is of great interest in the context of cancer therapy. However, cannabinoid receptor-mediated cell death has been observed in both normal and malignant immune cells (Guzman et al., 2002; McKallip et al., 2002b; Guzman, 2003; Do et al., 2004; Flygare et al., 2005; Powles et al., 2005). In the current study, *R*(+)-MA and Win55 induced apoptosis only in MCL cell lines and primary MCL, whereas normal B cells remained unaffected.

In conclusion, our study demonstrates that the cannabinoid receptor agonists *R*(+)-MA and Win55 induce a sequence of signaling events leading to cell death of MCL cells. The requirement of ligation of both CB_1 and CB_2 raises the possibility that cannabinoids may be used to selectively target MCL cells to undergo apoptosis.

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