Available online at www.sciencedirect.com
SCIENCE DIRECT
European Journal of Pharmacology 538 (2006) 154–162
www.elsevier.com/locate/ejphar

Suppression by WIN55212-2, a cannabinoid receptor agonist, of inflammatory reactions in mouse ear: Interference with the actions of an endogenous ligand, 2-arachidonoylglycerol

Saori Oka, Junichi Wakui, Maiko Gokoh, Seishi Kishimoto, Takayuki Sugiura *

Faculty of Pharmaceutical Sciences, Teikyo University, Sagamiko, Tsukui-gun, Kanagawa 199-0195, Japan

Received 24 October 2005; received in revised form 2 March 2006; accepted 13 March 2006
Available online 20 March 2006

Abstract

The effect of WIN55212-2, a cannabinoid receptor agonist, on acute inflammation of mouse ear was investigated. We found that topical application of WIN55212-2 suppressed ear swelling induced by 12-*O*-tetradecanoylphorbol 13-acetate or 2-arachidonoylglycerol. Similar inhibition was observed with CP55940, another cannabinoid receptor agonist, and HU-308, a cannabinoid CB_2 receptor-selective agonist. WIN55212-2 also suppressed the infiltration of leukocytes induced by 12-*O*-tetradecanoylphorbol 13-acetate. On the other hand, WIN55212-3, an inactive enantiomer of WIN55212-2, exerted only small effects on inflammation. Notably, SR144528, a cannabinoid CB_2 receptor antagonist, also suppressed inflammatory reactions in mouse ear. Thus, both the cannabinoid CB_2 receptor agonist and antagonist are capable of reducing inflammatory reactions. We then investigated the mechanism underlying WIN55212-2-induced suppression of inflammation using cultured cells. We found that the addition of WIN55212-2 together with 2-arachidonoylglycerol blocked 2-arachidonoylglycerol-induced migration of human promyelocytic leukemia HL-60 cells that had been differentiated into macrophage-like cells. The restoration of 2-arachidonoylglycerol-desensitized cells and WIN55212-2-desensitized cells from an anergic condition was examined next. We found that 2-arachidonoylglycerol-treated cells rapidly recovered the capacity to respond to 2-arachidonoylglycerol. On the other hand, the anergic condition toward 2-arachidonoylglycerol continued for a longer period after pretreatment with WIN55212-2. These results suggest that the anti-inflammatory activity of WIN55212-2 is attributable, at least in part, to interference with the actions of the endogenous ligand, 2-arachidonoylglycerol.
© 2006 Elsevier B.V. All rights reserved.

Keywords: Cannabinoid; 2-Arachidonoylglycerol; Cannabinoid receptor agonist; Cannabinoid receptor antagonist; Inflammation; Ear swelling

1. Introduction

Δ^9-Tetrahydrocannabinol (Δ^9-THC), a major psychoactive ingredient of marijuana, binds to specific receptors (cannabinoid receptors), thereby exhibiting a variety of pharmacological activities (Pertwee, 1997; Howlett et al., 2002). To date, two types of cannabinoid receptors have been identified. The cannabinoid CB_1 receptor is expressed abundantly in the nervous system, especially the brain (Matsuda et al., 1990), and has been assumed to play an important role in the attenuation of synaptic transmission (Pertwee, 1997; Di Marzo et al., 1998; Howlett et al., 2002). The cannabinoid CB_2 receptor is expressed mainly in various lymphoid tissues (Munro et al., 1993) and has been postulated to take part in the regulation of inflammatory reactions and immune responses (Pertwee, 1997; Howlett et al., 2002; Klein et al., 2003), but the details still remain rather ambiguous.

Previously, several investigators reported that the cannabinoid CB_2 receptor is involved in the modulation of chemokine production in HL-60 cells (Jbilo et al., 1999) and in the differentiation of B lymphocytes (Carayon et al., 1998). Buckley et al. (2000) demonstrated possible involvement of the cannabinoid CB_2 receptor in immunomodulatory functions of macrophages. We have focused on 2-arachidonoylglycerol (2-AG), an endogenous ligand for the cannabinoid receptors, and found that 2-AG induces a rapid, transient increase in $[Ca^{2+}]_i$ (Sugiura et al., 2000), the activation of p42/44 mitogen-activated protein kinase (MAP kinase) (Kobayashi et al., 2001) and

* Corresponding author. Fax: +81 426 85 1345.
E-mail address: sugiurat@pharm.teikyo-u.ac.jp (T. Sugiura).

0014-2999/$ - see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2006.03.021

augmented production of chemokines (Kishimoto et al., 2004) in HL-60 cells, and the migration of HL-60 cells differentiated into macrophage-like cells (Kishimoto et al., 2003), human peripheral blood monocytes (Kishimoto et al., 2003), natural killer cells (Kishimoto et al., 2005), and eosinophils (Oka et al., 2004) in cannabinoid CB_2 receptor-dependent manners. Several investigators also reported that 2-AG induces the migration of mouse splenocytes (Jorda et al., 2002) and microglia cells (Walter et al., 2003) via cannabinoid CB_2 receptor-dependent mechanisms. Notably, Iwamura et al. (2001) demonstrated that SR144528 and JTE-907, cannabinoid CB_2 receptor antagonists/inverse agonists, suppress carrageenan-induced mouse paw edema. Smith et al. (2001) also reported that SR144528 suppresses thioglycollate-induced peritoneal inflammation in mice. These observations strongly suggested that the cannabinoid CB_2 receptor and 2-AG are involved in the stimulation of several types of inflammatory reactions and immune responses. Recently, we obtained clear evidence that 2-AG and the cannabinoid CB_2 receptor play crucial stimulative roles in 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced acute inflammation of the mouse ear (Oka et al., 2005).

In contrast to the experimental results and the hypothesis mentioned above, several investigators reported that 2-AG and the cannabinoid CB_2 receptor are involved in the attenuation of inflammatory reactions and immune responses. Ouyang et al. (1998) demonstrated that 2-AG suppresses interleukin 2 (IL-2) secretion and inhibits IL-2 promoter activity. Chang et al. (2001) demonstrated that 2-AG suppresses the production of IL-6 in J774 macrophage-like cells, and Gallily et al. (2000) reported that 2-AG attenuates tumor-necrosis factor α (TNF-α) production in lipopolysaccharide-stimulated mouse macrophages. Yet, in some cases, it is not clear whether the effects of 2-AG are actually mediated via the cannabinoid CB_2 receptor. Several investigators also demonstrated that cannabinoid receptor agonists, such as WIN55212-2, HU-308, AM1241, CT-3, nabilone and anandamide, suppress inflammatory reactions in vivo (Burstein et al., 1992; Berdyshev et al., 1998; Richardson et al., 1998; Hanus et al., 1999; Smith et al., 2001; Clayton et al., 2002; Conti et al., 2002; Nackley et al., 2003; Pozzi et al., 2003; Quartilho et al., 2003; Di Filippo et al., 2004; Yoshihara et al., 2005). In most cases, the cannabinoid CB_2 receptor was suggested to be involved in inflammation, although there were some exceptions. The following question then arises: Does the cannabinoid CB_2 receptor participate in the stimulation of inflammation or the suppression of inflammation?

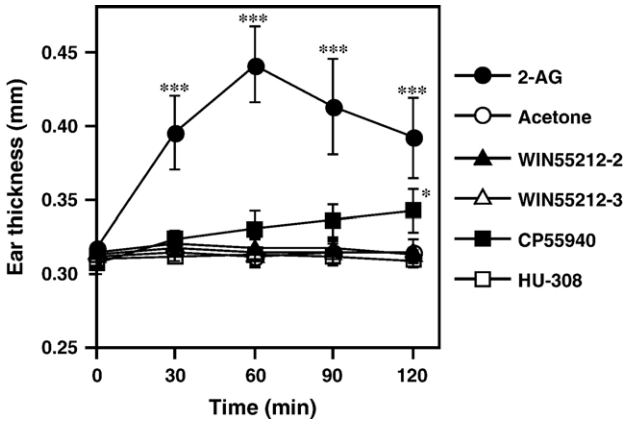

Fig. 1. Effects of the topical application of WIN55212-2, WIN55212-3, CP55940, HU-308 and 2-AG on mouse ear thickness. The effect of the topical application of WIN55212-2, WIN55212-3, CP55940, HU-308, or 2-AG (100 μg/ear each) or the vehicle (acetone) on mouse ear thickness was determined using a micrometer as described in Materials and methods. The data are the means ± S.D. of five determinations. $*P<0.05$, $***P<0.001$ [compared with control (acetone)].

The present investigation was undertaken to answer this question. In this study, we examined the effects of WIN55212-2 on TPA- or 2-AG-induced acute inflammation of the mouse ear and compared them with those of SR144528. We found that both WIN55212-2 and SR144528 suppress inflammatory reactions in mouse ear. The suppressive effect of WIN55212-2 was suggested to be due to interference with the actions of the endogenous ligand, 2-AG.

2. Materials and methods

2.1. Chemicals

Essentially fatty acid-free bovine serum albumin (BSA) was purchased from Sigma (St. Louis, MO). TPA (12-*O*-tetradecanoylphorbol 13-acetate) and Fura-2/AM were obtained from Wako Pure Chem. Ind. (Osaka, Japan). 2-AG and HU-308 (bicyclo[3.1.1]hept-2-ene-2-methanol,4-[4-(1,1-dimethylheptyl)-2-6-dimethoxyphenyl]-6,6-dimethyl-(1R,4R,5R)) were acquired from Cayman (Ann Arbor, MI, USA). WIN55212-2 ((R)-(1)[2,3-dihydro-5-methyl-3-[4-morpholino]methyl]pyrrolo-[1,2,3-de]-1,4-benzoxazin-6-yl)(1-naphthyl)methanone) and WIN55212-3 ((S)-(1)[2,3-dihydro-5-methyl-3-[4-morpholino]methyl]pyrrolo-[1,2,3-de]-1,4-benzoxazin-6-yl)(1-naphthyl)methanone) were obtained from RBI (Natick, MA, USA). CP55940 ((2)-3-[2-hydroxy-4-(1,1,-dimethylheptyl)phenyl]-4-(3-hydroxypropyl)cyclohexane-1-ol) was purchased from Tocris (Bristol, UK). SR144528 (*N*-([1s]-*endo*-1.3.3-trimethylbicyclo[2.2.1]heptan-2-yl)-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide) was a generous gift from Sanofi-Synthelabo Recherche (Montpellier, France).

2.2. Animals

Male ICR mice (body weight, 30 g) were obtained from Sankyo Labo Service (Tokyo, Japan). The present studies were conducted in accordance with the Guide for Care and Use of Laboratory Animals adopted by the Committee on Care and Use of Laboratory Animals of Teikyo University and the Japanese Pharmacological Society.

2.3. Effects of cannabinoid receptor ligands on TPA- or 2-AG-induced ear swelling

TPA (0.8 μg) or 2-AG (100 μg) dissolved in 20 μl of acetone was topically applied to the right ear of an ICR mouse. Control mice received the vehicle (acetone) alone. WIN55212-2,

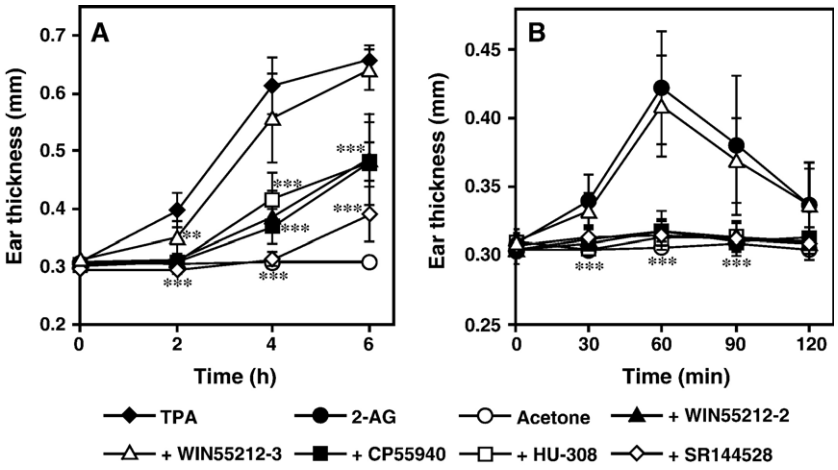

Fig. 2. Effects of WIN55212-2, WIN55212-3, CP55940, HU-308, and SR144528 on TPA- or 2-AG-induced ear swelling. (A) TPA (0.8 μg) or (B) 2-AG (100 μg) dissolved in acetone was topically applied to a mouse ear. Then, WIN55212-2, WIN55212-3, CP55940, HU-308, or SR144528 (30 μg each) dissolved in acetone was applied to the same ear. Ear thickness was measured using a micrometer. The data are the means ± S.D. of six determinations. $^{**}P<0.01$, $^{***}P<0.001$ [compared with TPA alone (A) or 2-AG alone (B)].

WIN55212-3, CP55940, HU-308, or SR144528 (30 μg each) dissolved in 20 μl of acetone was then applied to the same ear. In some experiments, one of several concentrations of WIN55212-2, CP55940, HU-308, or SR144528 was applied to the mouse ear immediately after the application of TPA. Ear thickness was measured using a micrometer (Mitutoyo, Japan) as described previously (Oka et al., 2005). The swelling rate was calculated using the following formula: (ear thickness after treatment − ear thickness before treatment) × 100 / ear thickness before treatment.

2.4. Measurement of myeloperoxidase activity

Myeloperoxidase (MPO), located in the azurophilic granules of neutrophils, has been used as an enzyme marker of neutrophil infiltration into inflamed tissues (Rawlingson et al., 2001). MPO activity of the mouse ear was estimated as described previously (Oka et al., 2005). In brief, the right ear of a TPA- or vehicle (acetone)-treated mouse was dissected, frozen in liquid nitrogen, and homogenized in 2 ml of 50 mM potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide, and centrifuged at 15,000 ×*g* for 20 min to collect supernatants to be used in subsequent MPO assays. The enzyme reaction of MPO was initiated by the addition of 100 μl of 3,3′,5,5′-tetramethylbenzenidine liquid substrate system (Sigma) to 10 μl of the sample in a 96-well plate. After standing at room temperature for 30 min, the reaction was stopped by the addition of 50 μl of 0.5 M H_2SO_4, and the absorbance at 450 nm was read using a microplate reader.

2.5. Cells

Human promyelocytic leukemia HL-60 cells were grown at 37 °C in RPMI 1640 medium (Asahi Technoglass Co., Chiba, Japan) supplemented with 10% fetal bovine serum (FBS) in an atmosphere of 95% air and 5% CO_2. HL-60 cells were

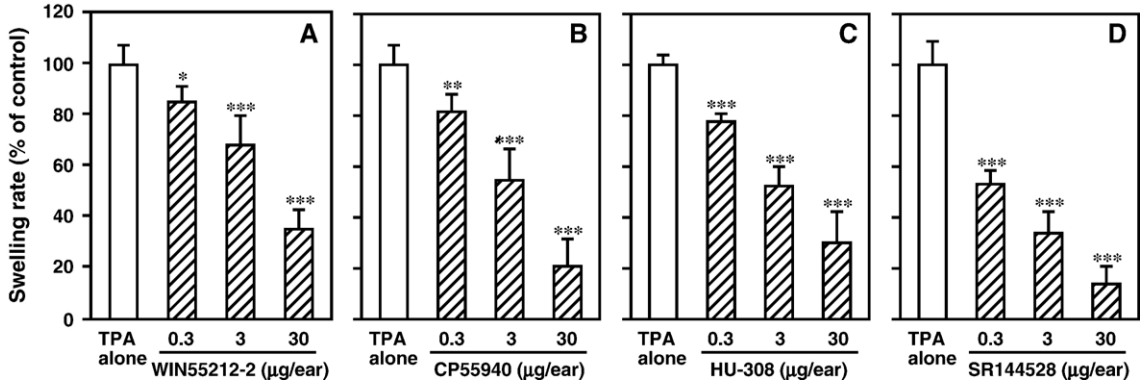

Fig. 3. Dose-dependencies of the effects of WIN55212-2, CP55940, HU-308, and SR144528 on TPA-induced ear swelling. TPA (0.8 μg) dissolved in acetone was topically applied to a mouse ear. Various concentrations of (A) WIN55212-2, (B) CP55940, (C) HU-308, or (D) SR144528 dissolved in acetone were then applied to the same ear. Four hours after the application of TPA and other compounds, ear thickness was measured using a micrometer. The swelling rate was calculated as described in Materials and methods and expressed as % of control (TPA alone). The data are the means ± S.D. of six determinations. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ [compared with control (TPA alone)].

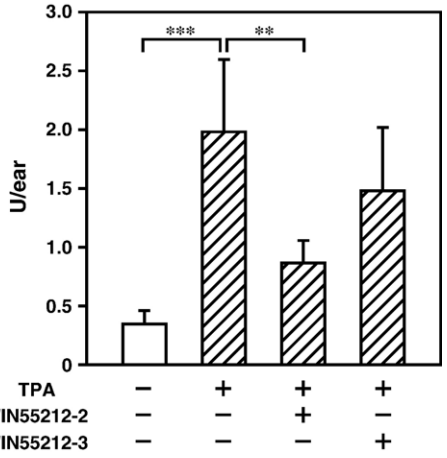

Fig. 4. Effects of WIN55212-2 and WIN55212-3 on the infiltration of neutrophils in TPA-treated mouse ear. TPA (0.8 μg) dissolved in acetone was topically applied to a mouse ear. Control mice received the vehicle (acetone) alone. WIN55212-2 or WIN55212-3 (30 μg each) or the vehicle (acetone) was then applied to the same ear. Six hours after the application of TPA and WIN55212-2 or WIN55212-3, the infiltration of neutrophils was determined by estimating MPO activity as described in Materials and methods. The data are the means±S.D. from six determinations. $^{**}P<0.01$, $^{***}P<0.001$.

differentiated into macrophage-like cells by treatment with 100 nM 1α,25-$(OH)_2$ vitamin D_3 for 5 days.

2.6. Migration assay

The migration of HL-60 cells that had been differentiated into macrophage-like cells was assayed using Transwell™ inserts (pore size, 5 μm) and 24-well culture plates (Corning Costar, Cambridge, MA) (Kishimoto et al., 2003). Briefly, 10^6 cells suspended in 0.1 ml of RPMI 1640 medium containing 0.1% BSA were transferred to the Transwell™ insert (upper compartment). 2-AG, WIN55212-2, or WIN55212-3 was dissolved in dimethyl sulfoxide (DMSO) and added to 0.6 ml of the RPMI 1640 medium containing 0.1% BSA in the well of the culture plate (lower compartment) (final concentration of each ligand, 1 μM; final concentration of DMSO, 0.4%, v/v). After incubation at 37 °C for 4 h in an atmosphere of 95% air and 5% CO_2, the number of cells that had migrated from the upper compartment to the lower compartment was counted using a hemocytometer.

2.7. Measurement of intracellular free Ca^{2+} concentrations ($[Ca^{2+}]_i$)

Subconfluent HL-60 cells that had been differentiated into macrophage-like cells were suspended by gentle pipetting in 25 mM HEPES-buffered Tyrode's solution ($-Ca^{2+}$) (pH 7.4) containing 3 μM Fura-2/AM and further incubated at 37 °C for 45 min. The cells were then centrifuged (180 ×*g* for 5 min), washed twice with HEPES–Tyrode's solution ($-Ca^{2+}$), and resuspended in 500 μl of HEPES–Tyrode's solution ($-Ca^{2+}$) containing 0.1% BSA (10^6 cells/ml). 2-AG, WIN55212-2, or WIN55212-3 was dissolved in DMSO, and an aliquot (1 μl) was added to the cell suspension (final concentration of each ligand, 3 μM; final concentration of DMSO, 0.2%, v/v). The incubation was carried out at 37 °C for 1 min. Cells were then sedimented by centrifugation, washed with HEPES–Tyrode's solution ($-Ca^{2+}$) containing 0.1% BSA, resuspended in the same buffer, and incubated at 37 °C for the indicated periods. $CaCl_2$ was added 2–3 min before the measurement of $[Ca^{2+}]_i$ (final concentration, 1 mM). After the addition of 2-AG to the cell suspension (final concentration, 1 μM), changes in $[Ca^{2+}]_i$ were analyzed using a CAF-100 Ca^{2+} analyzer (JASCO, Tokyo, Japan) (Sugiura et al., 2000).

2.8. Statistical analysis

The data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's test or Dunnett's test. Differences were considered significant at $P<0.05$.

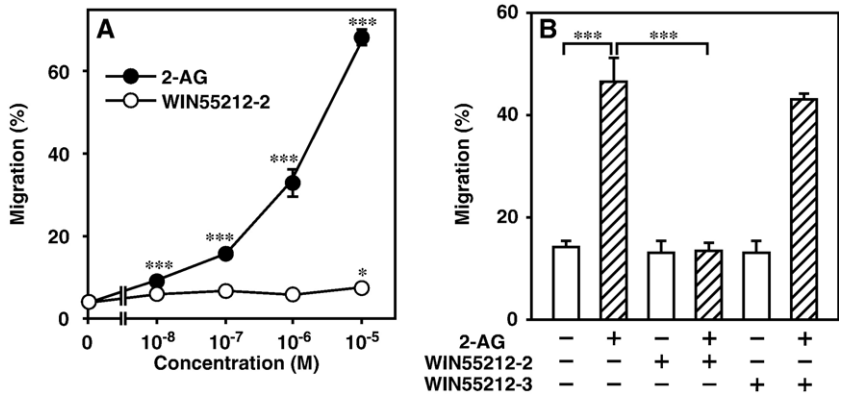

Fig. 5. Effects of WIN55212-2, WIN55212-3, and 2-AG on the motility of HL-60 cells differentiated into macrophage-like cells. (A) Various concentrations of 2-AG or WIN55212-2 were added to the lower compartment of Transwell™ inserts. The incubation was carried out for 4 h. The migration of HL-60 cells differentiated into macrophage-like cells from the upper to lower compartment was examined as described in Materials and methods. The data are the means±S.D. of four determinations. $^{*}P<0.05$, $^{***}P<0.001$ [compared with control (DMSO)]. (B) Effects of WIN55212-2 and WIN55212-3 on 2-AG-induced migration of HL-60 cells differentiated into macrophage-like cells. WIN55212-2, WIN55212-3, 2-AG (1 μM each), or the vehicle (DMSO) was added to the lower compartment. The incubation was carried out for 4 h. The migration of the cells from the upper to lower compartment was determined as described in Materials and methods. The data are the means±S.D. of four determinations. $^{***}P<0.001$.

3. Results

3.1. Effects of WIN55212-2, WIN55212-3, CP55940, HU-308, and 2-AG on mouse ear thickness

We first examined the effects of the topical application of several cannabinoid receptor ligands to mouse ear. As demonstrated in Fig. 1, the application of 2-AG (100 μg/ear) provoked ear swelling, the maximal effect being observed 1 h after the application of 2-AG. In contrast to 2-AG, other cannabinoid receptor ligands did not exert profound effects on ear thickness. The application of CP55940 (100 μg/ear) induced gradual swelling, yet the magnitude of the response was very small. Ear swelling was not apparent with 30 μg/ear of CP55940, while 30 μg/ear of 2-AG induced swelling (76% of that induced by 100 μg/ear of 2-AG at 1 h) (data not shown). No appreciable

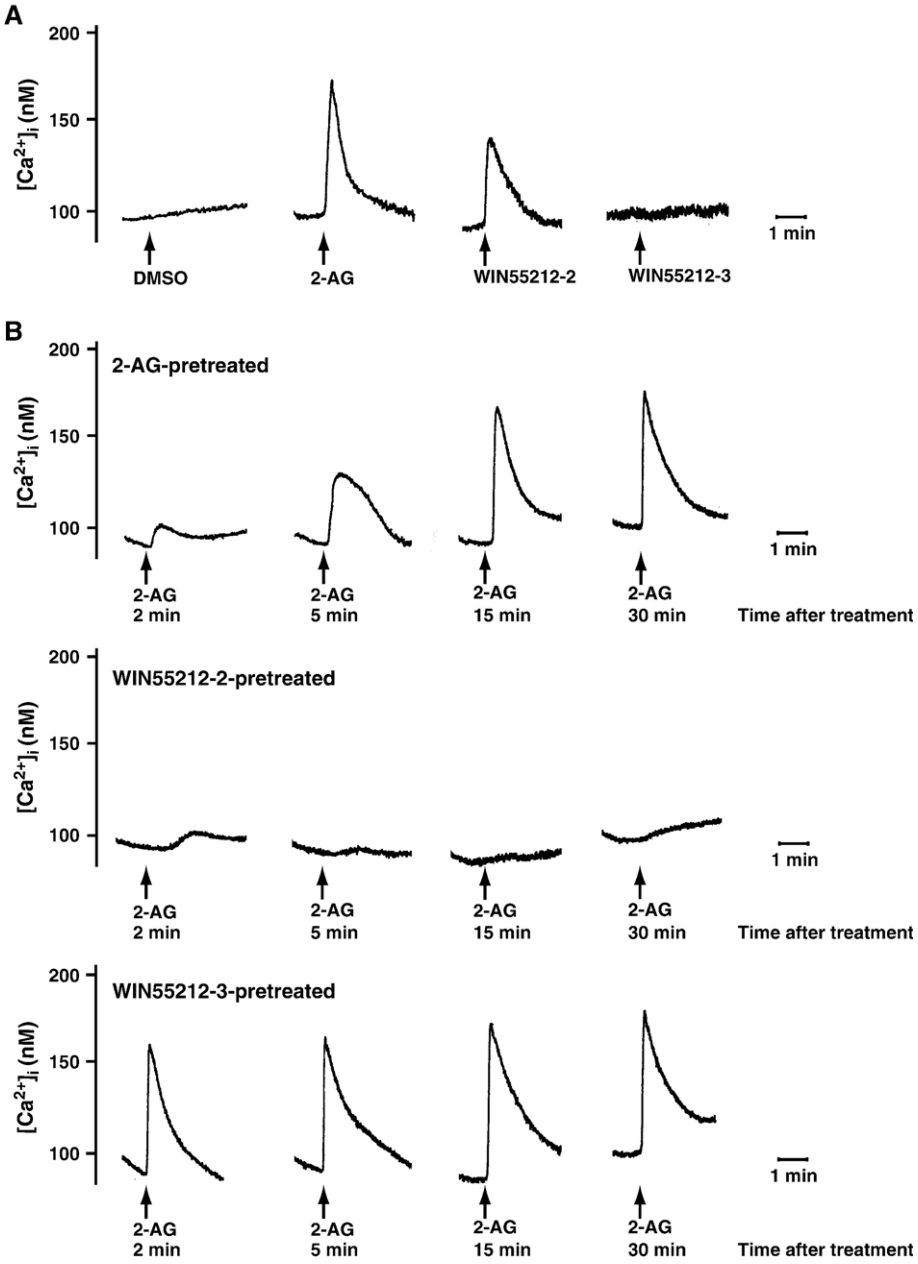

Fig. 6. Effects of WIN55212-2, WIN55212-3, and 2-AG on $[Ca^{2+}]_i$ in HL-60 cells differentiated into macrophage-like cells. Cells were loaded with Fura-2/AM at 37 °C for 45 min. Cells were then washed and resuspended in 25 mM HEPES–Tyrode's solution (pH 7.4). (A) WIN55212-2, WIN55212-3, and 2-AG were dissolved in DMSO and aliquots (1 μl each) were added to the cuvette. The final concentration of WIN55212-2, WIN55212-3 and 2-AG in the cuvette was 1 μM. Changes in $[Ca^{2+}]_i$ were estimated using a CAF-100 Ca^{2+} analyzer as described in Materials and methods. (B) Effects of pretreatment of the cells with 2-AG, WIN55212-2, and WIN55212-3 on 2-AG-induced elevation of $[Ca^{2+}]_i$. Cells were pretreated with 2-AG, WIN55212-2, or WIN55212-3 (3 μM each) for 1 min. Cells were then sedimented by centrifugation, washed with HEPES–Tyrode's solution (pH 7.4), and resuspended in 500 μl of HEPES–Tyrode's solution. Following incubation at 37 °C for indicated periods, 2-AG was added to the cuvette (final concentration, 1 μM). Changes in $[Ca^{2+}]_i$ were estimated using a CAF-100 Ca^{2+} analyzer as described in Materials and methods. The results are representative of three separate experiments, which gave similar results.

effect was observed with WIN55212-2 (100 μg/ear), WIN55212-3 (100 μg/ear), an inactive enantiomer of WIN55212-2, and HU-308, a cannabinoid CB_2 receptor-selective agonist (100 μg/ear).

3.2. Effects of WIN55212-2, WIN55212-3, CP55940, HU-308, and SR144528 on TPA- or 2-AG-induced ear swelling

We next examined the effects of the application of WIN55212-2, WIN55212-3, CP55940, HU-308, and SR144528 on TPA (0.8 μg/ear)- or 2-AG (100 μg/ear)-induced ear swelling. As demonstrated in Fig. 2A, WIN55212-2 (30 μg/ear), CP55940 (30 μg/ear), and HU-308 (30 μg/ear) markedly reduced TPA-induced ear swelling. SR144528 (30 μg/ear) was also shown to suppress ear swelling induced by TPA. On the other hand, WIN55212-3 (30 μg/ear) exerted only a small effect on TPA-provoked ear swelling.

Similar results were obtained for 2-AG-induced ear swelling (Fig. 2B). WIN55212-2 (30 μg/ear), CP55940 (30 μg/ear), HU-308 (30 μg/ear), and SR144528 (30 μg/ear) completely blocked 2-AG-induced ear swelling. In contrast to WIN55212-2, CP55940, HU-308, and SR144528, WIN55212-3 (30 μg/ear) did not affect 2-AG-elicited ear swelling.

Fig. 3 shows the dose dependencies of WIN55212-2, CP55940, HU-308, and SR144528 in the suppression of TPA-induced ear swelling. The IC_{50} values for WIN55212-2, CP55940, and HU-308 were 10.8, 4.0, and 3.7 μg/ear, respectively, which are 27, 10, and 9 times higher than that for SR144528 (0.4 μg/ear).

3.3. Effects of WIN55212-2 and WIN55212-3 on TPA-induced infiltration of leukocytes into mouse ear

We next examined the effects of WIN55212-2 and WIN55212-3 on TPA-induced infiltration of neutrophils. As depicted in Fig. 4, treatment of the ear with TPA evoked the infiltration of neutrophils into tissues, which was monitored by MPO activity. Notably, treatment of the ear with WIN55212-2 (30 μg/ear) markedly reduced the infiltration of neutrophils. In contrast to WIN55212-2, a marginal effect was observed with WIN55212-3 (30 μg/ear).

3.4. Effects of WIN55212-2, WIN55212-3, and 2-AG on cell migration

We then investigated the mechanism underlying WIN55212-2-induced suppression of inflammation. To simplify the experimental system, we examined the effects of WIN55212-2 on several cellular responses elicited by 2-AG in vitro. We first examined the effects of WIN55212-2 on 2-AG-induced migration of HL-60 cells that had been differentiated into macrophage-like cells. As demonstrated in Fig. 5A, 2-AG induced the migration of the cells from the upper compartment to the lower compartment of the Transwell™ inserts. In contrast to 2-AG, WIN55212-2 did not elicit apparent cell migration (Fig. 5A). Notably, the addition of WIN55212-2 (1 μM) together with 2-AG (1 μM) in the lower compartment completely blocked cell migration triggered by 2-AG, whereas WIN55212-3 (1 μM) did not affect 2-AG-induced cell migration (Fig. 5B).

3.5. Effects of WIN55212-2 and WIN55212-3 on 2-AG-induced Ca^{2+} transient

Finally, we examined the effects of pretreatment of the cells with 2-AG, WIN55212-2 and WIN55212-3 on 2-AG-induced elevation of $[Ca^{2+}]_i$ in HL-60 cells differentiated into macrophage-like cells. As shown in Fig. 6A, the addition of 2-AG or WIN55212-2 (1 μM) to the cells elicited a rapid increase in $[Ca^{2+}]_i$, indicating that both 2-AG and WIN55212-2 act as agonists toward the cannabinoid CB_2 receptor as has previously been reported (Sugiura et al., 2000). In contrast to 2-AG and WIN55212-2, WIN55212-3 (1 μM) failed to induce the elevation of $[Ca^{2+}]_i$.

The effects of pretreatment of the cells with 2-AG, WIN55212-2, and WIN55212-3 on 2-AG-induced elevation of $[Ca^{2+}]_i$ are depicted in Fig. 6B. 2-AG-pretreated cells responded to 2-AG very weakly at 2 min after pretreatment, presumably due to the desensitization of the receptor molecule. 2-AG-pretreated cells gradually recovered the capacity to respond to 2-AG (1 μM) with time. Complete recovery was observed 15 min after pretreatment. WIN55212-2-pretreated cells also did not respond to 2-AG (1 μM). Notably, the anergic condition continued at least 30 min after pretreatment, which is quite different from the case of 2-AG-pretreated cells described above. In contrast to 2-AG-pretreatment and WIN55212-2-pretreatment, WIN55212-3-pretreatment did not affect the cellular response to 2-AG.

4. Discussion

Recently, we investigated the possible involvement of the cannabinoid CB_2 receptor and 2-AG in TPA-induced acute inflammation in mouse ear (Oka et al., 2005). We found that the amount of 2-AG in mouse ear was markedly augmented following the application of TPA. Notably, TPA-induced ear swelling was blocked by treatment with SR144528 but not with AM251, a cannabinoid CB_1 receptor antagonist. The application of SR144528 also reduced the TPA-induced leukotriene B_4 production and infiltration of neutrophils in mouse ear. Importantly, the topical application of 2-AG to mouse ear evoked swelling, which was abolished by treatment with SR144528. AM251 did not affect 2-AG-induced ear swelling. These results strongly suggested that the cannabinoid CB_2 receptor and 2-AG play crucial stimulative roles during the course of inflammatory reactions. In addition to this, we recently obtained evidence that the cannabinoid CB_2 receptor and 2-AG are closely involved in oxazolone-induced contact dermatitis in mouse ear (Oka, S. and Sugiura, T., unpublished results). These results are consistent with the results reported by Iwamura et al. (2001) that several cannabinoid CB_2 receptor antagonists/inverse agonists suppress carrageenan-induced inflammation of the mouse paw. However, our results (Oka et al., 2005) and those by Iwamura et al. (2001) appear to be in conflict with the results of several investigators that cannabinoid

receptor agonists such as WIN55212-2, HU-308, AM1241, CT-3, nabilone, and anandamide suppress inflammatory reactions in vivo (Burstein et al., 1992; Berdyshev et al., 1998; Richardson et al., 1998; Hanus et al., 1999; Clayton et al., 2002; Conti et al., 2002; Nackley et al., 2003; Pozzi et al., 2003; Quartilho et al., 2003; Di Filippo et al., 2004; Yoshihara et al., 2005).

Which type of receptor ligand then actually suppresses inflammation, antagonist or agonist? To answer this question, we examined the effects of WIN55212-2 on acute inflammation in mouse ear and compared them with those of SR144528. To minimize the central nervous system-mediated effects, we adopted topical application as the administration route. We confirmed that SR144528 suppressed inflammatory reactions in mouse ear (Figs. 2 and 3). Notably, the application of WIN55212-2 to mouse ear also reduced inflammatory reactions (Figs. 2, 3 and 4), although the activity of WIN55212-2 was somewhat lower than that of SR144528 (Fig. 3). It is evident that the cannabinoid receptors are involved in WIN55212-2-induced suppression, because WIN55212-3, an inactive enantiomer of WIN55212-2, exerted only small effects on inflammatory reactions (Figs. 2, 3 and 4). Moreover, a similar and slightly more pronounced inhibition was observed with CP55940, another cannabinoid receptor agonist (Figs. 2 and 3). It seems likely that the cannabinoid CB_2 receptor is involved in the suppression, because HU-308, a cannabinoid CB_2 receptor-selective agonist, exhibited a suppressing activity comparable to those of WIN55212-2 and CP55940 (Figs. 2 and 3). It thus became apparent that both the cannabinoid CB_2 receptor agonist and antagonist/inverse agonist are more or less capable of reducing inflammatory reactions. This is a rather surprising observation because agonists and antagonists usually act oppositely, and the biological activities of various agonists are usually nullified by specific antagonists.

Then, how did these cannabinoid receptor agonists and antagonists suppress inflammatory reactions? Inasmuch as WIN55212-2 suppressed 2-AG-induced ear swelling (Figs. 2 and 3), we focused on the effects of WIN55212-2 on 2-AG-elicited cellular responses. As demonstrated in Fig. 5A, WIN55212-2 did not induce the migration of differentiated HL-60 cells, which express the cannabinoid CB_2 receptor (Munro et al., 1993; Sugiura et al., 2000); instead, WIN55212-2 blocked the cell migration induced by 2-AG (Fig. 5B). We have previously demonstrated that SR144528 blocked 2-AG-induced cell migration (Kishimoto et al., 2003), similar to the case of WIN55212-2 shown in the present study. Both 2-AG and WIN55212-2 act as agonists toward the cannabinoid CB_2 receptor (Pertwee, 1997; Sugiura et al., 2000; Howlett et al., 2002). Why was WIN55212-2 incapable of inducing cell migration, while 2-AG potently induced it? A possible explanation is as follows: Continuous stimulation is necessary for certain types of cellular response involving complex processes, such as cell migration. In contrast to 2-AG, continuous stimulation is not feasible with WIN55212-2, as described below.

The desensitization of the receptor molecule occurred in both 2-AG-pretreated cells and WIN55212-2-pretreated cells (Fig. 6B). Notably, the recovery from an anergic condition occurred rapidly after pretreatment with 2-AG (Fig. 6B). This may be attributed, at least in part, to the fact that 2-AG is a structurally flexible and metabolically labile molecule. Probably, 2-AG is rapidly removed from the receptor molecule and degraded by hydrolyzing enzymes (Dinh et al., 2002). On the other hand, WIN55212-2 is a stable and rigid molecule and may bind tightly to the receptor molecule. It appeared that much more time is required to eliminate WIN55212-2 from the binding site and recycle the receptor molecule, as compared with the case of 2-AG. Presumably due to this, the anergic condition is sustained for a longer period in the case of WIN55212-2 (Fig. 6B). This is a possible reason why WIN55212-2 interferes with the action of 2-AG and behaves as almost an antagonist in some cases, such as in vivo (Figs. 2, 3 and 4) and prolonged incubation experiments in vitro (Fig. 5).

There is growing evidence suggesting that 2-AG plays an essential role in the stimulation of inflammatory reactions and immune responses by acting on the cannabinoid CB_2 receptor (Sugiura et al., 2000; Kobayashi et al., 2001; Jorda et al., 2002; Walter et al., 2003; Kishimoto et al., 2003, 2004, 2005; Oka et al., 2004, 2005). Consequently, whether it is a cannabinoid CB_2 receptor antagonist or agonist, a molecule that interferes with the actions of 2-AG suppresses inflammatory reactions. Thus, the observation that the cannabinoid CB_2 receptor agonists reduce inflammation (Figs. 2, 3 and 4) and the observation that the CB_2 receptor antagonists suppress inflammatory reactions (Figs. 2 and 3) (Oka et al., 2005) are not necessarily contradictory. A similar paradoxical observation has been reported for a sphingosine-1-phosphate (S1P) receptor and its ligand. Matloubian et al. (2004) demonstrated that FTY720, a S1P receptor agonist, inhibits lymphocyte egress from secondary lymphoid tissues, which is also observed with S1P receptor ($S1P_1$)–knockout mice. The inhibitory action of FTY720 in vivo has been assumed to be due to the down-regulation of the receptor molecule (Matloubian et al., 2004; Chiba, 2005).

Finally, it is necessary to mention the following. In the previous studies where the effects of the cannabinoid receptor ligands on inflammation were examined, several investigators administered non-selective cannabinoid receptor agonists such as WIN55212-2 systemically. In that case, systemic effects such as the central nervous system-mediated effects cannot be avoided. In fact, Smith et al. (2001) reported that at least a part of the anti-inflammatory actions of cannabinoid receptor agonists administered systemically was attributable to central effects. Several investigators also injected cannabinoid receptor agonists locally. Interestingly, the anti-inflammatory actions of locally injected cannabinoid CB_2 receptor agonists were counteracted by CB_2 receptor antagonists such as SR144528 and AM630 (Hanus et al., 1999; Clayton et al., 2002; Conti et al., 2002; Nackley et al., 2003; Quartilho et al., 2003; Di Filippo et al., 2004; Yoshihara et al., 2005). In contrast, however, we did not observe such antagonism between WIN55212-2 and SR144528 when WIN55212-2 (30 μg/ear) and SR144528 (30 μg/ear) were topically applied to mouse ear (data not shown). The reason for such a difference is not yet clear. It does not appear to be due to different absorption rates for cannabinoid

receptor agonists and antagonists; the topical administration of SR144528 to mouse ear markedly reduced ear swelling induced by TPA as in the cases of the administration of WIN55212-2, CP55940 and HU-308 (Figs. 2 and 3). Inflammation is a complex biological process involving a variety of pathophysiological events such as edema, redness, heat, and pain. These responses are known to be regulated by diverse biological systems including the immune system, vascular system, peripheral nervous system, and the central nervous system in complex and sophisticated manners. Further detailed studies are thus essential for a thorough elucidation of the mechanism underlying cannabinoid receptor agonist-mediated suppression of inflammation in vivo.

In conclusion, we found that both the cannabinoid CB_2 receptor agonist and the CB_2 receptor antagonist suppress inflammatory reactions in mouse ear when applied topically. The suppression was assumed to be due to interference with the actions of the endogenous ligand 2-AG in either case. These observations strongly suggest that not only the cannabinoid CB_2 receptor antagonist, but also the CB_2 receptor agonist, is a potential target in the development of a novel class of anti-inflammatory drugs and immune-suppressive agents.

References

Berdyshev, E., Boichot, E., Corbel, M., Germain, N., Lagente, V., 1998. Effects of cannabinoid receptor ligands on LPS-induced pulmonary inflammation in mice. Life Sci. 63, PL125-9.

Buckley, N.E., McCoy, K.L., Mezey, E., Bonner, T., Zimmer, A., Felder, C.C., Glass, M., Zimmer, A., 2000. Immunomodulation by cannabinoids is absent in mice deficient for the cannabinoid CB(2) receptor. Eur. J. Pharmacol. 396, 141–149.

Burstein, S.H., Audette, C.A., Breuer, A., Devane, W.A., Colodner, S., Doyle, S.A., Mechoulam, R., 1992. Synthetic nonpsychotropic cannabinoids with potent antiinflammatory, analgesic, and leukocyte antiadhesion activities. J. Med. Chem. 35, 3135–3141.

Carayon, P., Marchand, J., Dussossoy, D., Derocq, J.M., Jbilo, O., Bord, A., Bouaboula, M., Galiegue, S., Mondiere, P., Penarier, G., Le Fur, G., Defrance, T., Casellas, P., 1998. Modulation and functional involvement of CB2 peripheral cannabinoid receptors during B-cell differentiation. Blood 92, 3605–3615.

Chang, Y.H., Lee, S.T., Lin, W.W., 2001. Effects of cannabinoids on LPS-stimulated inflammatory mediator release from macrophages: involvement of eicosanoids. J. Cell. Biochem. 81, 715–723.

Chiba, K., 2005. FTY720, a new class of immunomodulator, inhibits lymphocyte egress from secondary lymphoid tissues and thymus by agonistic activity at sphingosine 1-phosphate receptors. Pharmacol. Ther. 108, 308–319.

Clayton, N., Marshall, F.H., Bountra, C., O'Shaughnessy, C.T., 2002. CB1 and CB2 cannabinoid receptors are implicated in inflammatory pain. Pain 96, 253–260.

Conti, S., Costa, B., Colleoni, M., Parolaro, D., Giagnoni, G., 2002. Antiinflammatory action of endocannabinoid palmitoylethanolamide and the synthetic cannabinoid nabilone in a model of acute inflammation in the rat. Br. J. Pharmacol. 135, 181–187.

Di Filippo, C., Rossi, F., Rossi, S., D'Amico, M., 2004. Cannabinoid CB_2 receptor activation reduces mouse myocardial ischemia–reperfusion injury: involvement of cytokine/chemokines and PMN. J. Leukoc. Biol. 75, 453–459.

Di Marzo, V., Melck, D., Bisogno, T., De Petrocellis, L., 1998. Endocannabinoids, endogenous cannabinoid receptor ligands with neuromodulatory action. Trends Neurosci. 21, 521–528.

Dinh, T.P., Freund, T.F., Piomelli, D., 2002. A role for monoglyceride lipase in 2-arachidonoylglycerol inactivation. Chem. Phys. Lipids 121, 149–158.

Gallily, R., Breuer, A., Mechoulam, R., 2000. 2-Arachidonylglycerol, an endogenous cannabinoid, inhibits tumor necrosis factor-α production in murine macrophages, and in mice. Eur. J. Pharmacol. 406, R5–R7.

Hanus, L., Breuer, A., Tchilibon, S., Shiloah, S., Goldenberg, D., Horowitz, M., Pertwee, R.G., Ross, R.A., Mechoulam, R., Fride, E., 1999. HU-308: a specific agonist for CB_2, a peripheral cannabinoid receptor. Proc. Natl. Acad. Sci. U. S. A. 96, 14228–14233.

Howlett, A.C., Barth, F., Bonner, T.I., Cabral, G., Casellas, P., Devane, W.A., Felder, C.C., Herkenham, M., Mackie, K., Martin, B.R., Mechoulam, R., Pertwee, R.G., International Union of Pharmacology, 2002. XXVII. Classification of cannabinoid receptors. Pharmacol. Rev. 54, 161–202.

Iwamura, H., Suzuki, H., Ueda, Y., Kaya, T., Inaba, T., 2001. In vitro and in vivo pharmacological characterization of JTE-907, a novel selective ligand for cannabinoid CB_2 receptor. J. Pharmacol. Exp. Ther. 296, 420–425.

Jbilo, O., Derocq, J.M., Segui, M., Le Fur, G., Casellas, P., 1999. Stimulation of peripheral cannabinoid receptor CB_2 induces MCP-1 and IL-8 gene expression in human promyelocytic cell line HL60. FEBS Lett. 448, 273–277.

Jorda, M.A., Verbakel, S.E., Valk, P.J., Vankan-Berkhoudt, Y.V., Maccarrone, M., Finazzi-Agro, A., Lowenberg, B., Delwel, R., 2002. Hematopoietic cells expressing the peripheral cannabinoid receptor migrate in response to the endocannabinoid 2-arachidonoylglycerol. Blood 99, 2786–2793.

Kishimoto, S., Gokoh, M., Oka, S., Muramatsu, M., Kajiwara, T., Waku, K., Sugiura, T., 2003. 2-Arachidonoylglycerol induces the migration of HL-60 cells differentiated into macrophage-like cells and human peripheral blood monocytes through the cannabinoid CB_2 receptor-dependent mechanism. J. Biol. Chem. 278, 24469–24475.

Kishimoto, S., Kobayashi, Y., Oka, S., Gokoh, M., Waku, K., Sugiura, T., 2004. 2-Arachidonoylglycerol, an endogenous cannabinoid receptor ligand, induces accelerated production of chemokines in HL-60 cells. J. Biochem. 135, 517–524.

Kishimoto, S., Muramatsu, M., Gokoh, M., Oka, S., Waku, K., Sugiura, T., 2005. Endogenous cannabinoid receptor ligand induces the migration of human natural killer cells. J. Biochem. 137, 217–223.

Klein, T.W., Larsen, C.K., Lu, L., Perkins, I., Nong, L., Friedman, H., 2003. The cannabinoid system and immune modulation. J. Leukoc. Biol. 74, 486–496.

Kobayashi, Y., Arai, S., Waku, K., Sugiura, T., 2001. Activation by 2-arachidonoylglycerol, an endogenous cannabinoid receptor ligand, of p42/44 mitogen-activated protein kinase in HL-60 cells. J. Biochem. 129, 665–669.

Matloubian, M., Lo, C.G., Cinamon, G., Lesneski, M.J., Xu, Y., Brinkmann, V., Allende, M.L., Proia, R.L., Cyster, J.G., 2004. Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. Nature 427, 355–360.

Matsuda, L.A., Lolait, S.J., Brownstein, M.J., Young, A.C., Bonner, T.I., 1990. Structure of a cannabinoid receptor and functional expression of the cloned cDNA. Nature 346, 561–564.

Munro, S., Thomas, K.L., Abu-Shaar, M., 1993. Molecular characterization of a peripheral receptor for cannabinoids. Nature 365, 61–65.

Nackley, A.G., Suplita II, R.L., Hohmann, A.G., 2003. A peripheral cannabinoid mechanism suppresses spinal fos protein expression and pain behavior in a rat model of inflammation. Neuroscience 117, 659–670.

Oka, S., Ikeda, S., Kishimoto, S., Gokoh, M., Yanagimoto, S., Waku, K., Sugiura, T., 2004. 2-Arachidonoylglycerol, an endogenous cannabinoid receptor ligand, induces the migration of EoL-1 human eosinophilic leukemia cells and human peripheral blood eosinophils. J. Leukoc. Biol. 76, 1002–1009.

Oka, S., Yanagimoto, S., Ikeda, S., Gokoh, M., Kishimoto, S., Waku, K., Ishima, Y., Sugiura, T., 2005. Evidence for the involvement of the cannabinoid CB_2 receptor and its endogenous ligand 2-arachidonoylglycerol in 12-*O*-tetradecanoylphorbol-13-acetate-induced acute inflammation in mouse ear. J. Biol. Chem. 280, 18488–18497.

Ouyang, Y., Hwang, S.G., Han, S.H., Kaminski, N.E., 1998. Suppression of interleukin-2 by the putative endogenous cannabinoid 2-arachidonylglycerol is mediated through down-regulation of the nuclear factor of activated T cells. Mol. Pharmacol. 53, 676–683.

Pertwee, R.G., 1997. Pharmacology of cannabinoid CB_1 and CB_2 receptors. Pharmacol. Ther. 74, 129–180.

Pozzi, O., Misiano, P., Clark, G.D., Visentin, L., 2003. Antagonism between the anti-inflammatory activity of the cannabinoid WIN 55212-2 and SR 141716A. Pharmacology 69, 158–163.

Quartilho, A., Mata, H.P., Ibrahim, M.M., Vanderah, T.W., Porreca, F., Makriyannis, A., Malan Jr., T.P., 2003. Inhibition of inflammatory

hyperalgesia by activation of peripheral CB_2 cannabinoid receptors. Anesthesiology 99, 955–960.

Rawlingson, A., Gerard, N.P., Brain, S.D., 2001. Interactive contribution of NK_1 and kinin receptors to the acute inflammatory oedema observed in response to noxious heat stimulation: studies in NK_1 receptor knockout mice. Br. J. Pharmacol. 134, 1805–1813.

Richardson, J.D., Kilo, S., Hargreaves, K.M., 1998. Cannabinoids reduce hyperalgesia and inflammation via interaction with peripheral CB_1 receptors. Pain 75, 111–119.

Smith, S.R., Denhardt, G., Terminelli, C., 2001. The anti-inflammatory activities of cannabinoid receptor ligands in mouse peritonitis models. Eur. J. Pharmacol. 432, 107–119.

Sugiura, T., Kondo, S., Kishimoto, S., Miyashita, T., Nakane, S., Kodaka, T., Suhara, Y., Takayama, H., Waku, K., 2000. Evidence that 2-arachidonoylglycerol but not *N*-palmitoylethanolamine or anandamide is the physiological ligand for the cannabinoid CB_2 receptor. Comparison of the agonistic activities of various cannabinoid receptor ligands in HL-60 cells. J. Biol. Chem. 275, 605–612.

Walter, L., Franklin, A., Witting, A., Wade, C., Xie, Y., Kunos, G., Mackie, K., Stella, N., 2003. Nonpsychotropic cannabinoid receptors regulate microglial cell migration. J. Neurosci. 23, 1398–1405.

Yoshihara, S., Morimoto, H., Ohori, M., Yamada, Y., Abe, T., Arisaka, O., 2005. The cannabinoid receptor agonist WIN 55212-2 inhibits neurogenic inflammations in airway tissues. J. Pharmacol. Sci. 98, 77–82.