Rapid Communication *J. Biochem.* **129**, 665–669 (2001)

Activation by 2-Arachidonoylglycerol, an Endogenous Cannabinoid Receptor Ligand, of p42/44 Mitogen-Activated Protein Kinase in HL-60 Cells[1]

Yuriko Kobayashi, Shunsuke Arai, Keizo Waku, and Takayuki Sugiura[2]

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

Received February 13, 2001; accepted March 5, 2001

2-Arachidonoylglycerol (2-AG), an endogenous cannabinoid receptor ligand, was shown to induce rapid phosphorylation of p42/44 mitogen-activated protein kinase (MAP kinase) in HL-60 cells. We confirmed that the enzyme activity of p42/44 MAP kinase in HL-60 cells was augmented markedly when the cells were stimulated with 2-AG. The addition of SR144528, a cannabinoid CB2 receptor-specific antagonist, to the cells prior to the addition of 2-AG abolished the response induced by 2-AG, indicating that the CB2 receptor is involved in the response. G protein G_i or G_o is also assumed to be involved, because pertussis toxin treatment of the cells nullified the response induced by 2-AG. CP55940 and anandamide also induced the activation of p42/44 MAP kinase, although the activation by anandamide was less pronounced than that by 2-AG or CP55940. These results suggest that 2-AG may play an important physiological role in this type of cell through the activation of the p42/44 MAP kinase cascade.

Key words: anandamide, 2-arachidonoylglycerol, cannabinoid, CB2 receptor, MAP kinase.

Δ^9-Tetrahydrocannabinol, a major psychoactive ingredient of marijuana, is known to bind to specific binding sites, *i.e.*, cannabinoid receptors, thereby eliciting diverse pharmacological responses in experimental animals and man (*1*). Previously, we (*2*) and Mechoulam *et al.* (*3*) demonstrated that 2-arachidonoylglycerol (2-AG), a unique molecular species of monoacylglycerol, is a possible endogenous ligand for the cannabinoid receptors. 2-AG binds to both central and peripheral cannabinoid receptors (CB1 and CB2)(*2*, *3*), and induces a variety of biological responses including analgesia (*3*), hypothermia (*3*), reduced spontaneous activity (*3*), hypotension (*4*, *5*), induction of Ca^{2+} transients in NG108-15 cells and HL-60 cells (*6–9*), inhibition of adenylyl cyclase (*3*), inhibition of vas deferens constriction (*3*), modulation of lymphocyte proliferation (*10*, *11*), inhibition of long-term potentiation in hippocampal slices (*12*), and inhibition of depolarization-induced elevation of the intracellular free Ca^{2+} concentration in neural cells (*13*). Nonetheless, available information concerning 2-AG is limited compared with that on anandamide, another endogenous cannabinoid receptor ligand. Details of the physiological significance and biological activities of 2-AG remain obscure.

Recently, we examined in detail the structure-activity relationship of various cannabinoid receptor agonists by measuring their capacities to induce Ca^{2+} transients in NG108-15 cells, which express the CB1 receptor (*6–8*), and in HL-60 cells, which express the CB2 receptor (*9*). We found that anandamide is a weak partial agonist toward either the CB1 receptor or the CB2 receptor (*6–9*). In contrast, 2-AG was found to act as a potent full agonist at both types of cannabinoid receptors (*6–9*). Based on these results, we proposed that 2-AG, but not anandamide, is the natural ligand for both the CB1 and CB2 receptors, and that these cannabinoid receptors are primarily 2-AG receptors (*7–9*). Further intensive studies on 2-AG are therefore essential to clarify the physiological and pathophysiological roles of the cannabinoid receptor system in mammalian tissues.

In this study, we examined whether or not 2-AG induces the activation of p42/44 mitogen-activated protein kinase (MAP kinase), which is known to play essential roles in the intracellular signal transduction of stimulated cells.

Arachidonic acid and essentially fatty acid-free bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO, USA). 2-Aminoethanol (ethanolamine) was obtained from Tokyo Kasei (Tokyo). 2-AG and anandamide were chemically synthesized from arachidonic acid and glycerol, and arachidonic acid and 2-aminoethanol, respectively, as described previously (*8*). CP55940 was purchased from Tocris (Bristol, UK). SR144528 was a generous gift from Sanofi (Montpellier, France). Pertussis toxin (PTX) was obtained from List Biological Laboratories (Campbell, CA, USA). PD98059 was purchased from Calbiochem (San Diego, CA, USA). A mouse antibody specific to phosphorylated p42/44 MAP kinase (Thr202/Tyr204) was obtained from RBI (Natic, MA, USA). A biotinylated rat anti-mouse IgG antibody was from Funakoshi (Tokyo). ABC (avidin: biotinylated enzyme complex) reagent was from Vector

[1] This study was supported in part by Grants-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan, and the Uehara Memorial Foundation.

[2] To whom correspondence should be addressed. Fax: 81-426-85-1345, E-mail: sugiurat@pharm.teikyo-u.ac.jp

Abbreviations: MAP kinase, mitogen-activated protein kinase; 2-AG, 2-arachidonoylglycerol; PTX, pertussis toxin; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide.

Laboratories, Inc. (Burlingame, CA, USA). A rabbit antibody specific to MAP kinase was obtained from Zymed Laboratories (So. San Francisco, CA, USA). A goat anti-rabbit IgG antibody was purchased from MBL (Nagoya). ECL reagent was obtained from Amersham Pharmacia Biotech. (Piscataway, NJ, USA). A p42/44 MAP kinase assay kit was acquired from Cell Signaling Technology (Beverly, MA, USA).

HL-60 cells were grown at 37°C in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) under an atmosphere of 95% air and 5% CO_2. Western blot analysis of phosphorylated p42/44 MAP kinase was carried out as follows. Subconfluent cells were incubated in fresh medium without FBS for 24 h. The cells were then spun down and resuspended in RPMI 1640 containing 0.1% BSA at 8×10^6 cells/ml. 2-AG dissolved in 1 μl of dimethyl sulfoxide (DMSO) was added to 500 μl of the prewarmed cell suspension. The incubation was carried out at 37°C for 0.5–5 min. Then the cell suspension was centrifuged at 15,000 ×*g* for 1 min. The sedimented cells were lysed with 150 μl of lysis buffer composed of 20 mM Hepes-NaOH (pH 7.3), 1% Triton X-100, 10% glycerol, 1 mM EDTA, 50 mM sodium fluoride, 2.5 mM *p*-nitrophenyl phosphate, 0.05 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate and 10 μg/ml of leupeptin. The cell lysate was centrifuged at 15,000 ×*g* for 20 min. The supernatant was aspirated into another tube, and then 60 μl of sample buffer containing 230 mM Tris-HCl (pH 6.8), 10% SDS, 25% glycerol, 25% 2-mercaptoethanol, and 0.1% bromophenol blue was added and the tube was heated at 100°C for 5 min. The sample was fractionated by SDS/PAGE on 8% gels. The separated fractions were electrotransferred to a nitrocellulose membrane, and then blocked by incubation in a blocking solution composed of 0.1% Tween 20 in 40 mM Tris-HCl buffered saline (TBST) (pH 7. 5) containing 5% BSA for 1 h. The blots were treated with the anti-p42/44 phosphorylated MAP kinase antibody (at 1:2,500 dilution) overnight at 4°C. The membranes were washed with TBST and then treated with the biotinylated anti–mouse IgG secondary antibody (at 1:4,000 dilution) in TBST containing 5% BSA at 4°C for 3 h. After further washing with TBST, the membranes were treated with the ABC reagent at room temperature for 0.5 h. The membranes were then washed, treated with the ECL reagent and exposed to X-ray films.

Western blot analysis of p42/44 MAP kinase was carried out as follows. Membranes were incubated in stripping buffer composed of 20 mM Tris-HCl (pH 7.4), 8 M guanidine hydrochloride, and 10 mM 2-mercaptoethanol at room temperature for 30 min to strip the used antibody off the membranes. After incubation, the membranes were blocked with the blocking solution, and then an immunoblot reaction was performed with the primary antibody, anti-p42/44 MAP kinase antibody (at 1:2,000 dilution), followed by the secondary antibody, anti–rabbit IgG antibody (at 1:2,000 dilution). The membranes were washed, treated with the ECL reagent and exposed to X-ray films.

p42/44 MAP kinase activity was measured with the p42/44 MAP kinase assay kit using a transcription factor Elk-1 fusion protein as a substrate. Briefly, 200 μl of a cell lysate prepared as described above was mixed with beads coated with the mouse antibody to phosphorylated p42/44 MAP kinase (Thr202/Tyr204), and the gently agitated at 4°C for 4 h. The mixture was centrifuged at 15,000 ×*g* for 1 min. The pellet was washed four times and then suspended in 50 μl of 25 mM Tris-HCl buffer (pH 7.5) containing 10 mM $MgCl_2$, 5 mM β-glycerophosphate, 2 mM dithiothreitol, 0.1 mM sodium orthovanadate, 200 mM ATP, and 2 μg of Elk-1 fusion protein. The enzyme reaction was carried out at 30°C for 30 min. The incubation was stopped by the addition of 12.5 μl of sample buffer composed of 125 mM Tris-HCl (pH 6.8), 10% SDS, 25% glycerol, 25% 2-mercaptoethanol, and 0.1% bromophenol blue. The mixture was then heated at 100°C for 5 min. The sample was fractionated by SDS/PAGE on 10% gels. The separated fractions were electrotransferred to a nitrocellulose membrane, and then blocked with the blocking solution. The blots were treated with the rabbit anti–phosphorylated Elk-1 protein antibody (at 1:1,000 dilution) overnight at 4°C. The membranes were washed with TBST and then treated with the horseradish peroxidase–conjugated anti–rabbit IgG antibody (at 1:2,000 dilution) in the blocking solution at 4°C for 3 h. After washing with TBST, the membranes were treated with Lumi GLO reagent at room temperature for 1 min. The membranes were then washed and exposed to X-ray films. The intensity of bands was quantified using NIH *Image*.

First, we examined the effect of 2-AG on p42/44 MAP kinase in HL-60 cells by Western blot analysis using the anti-phosphorylated p42/44 MAP kinase antibody. Figure 1 shows a representative result of such analysis. We found that the addition of 1 μM 2-AG to the cells triggers rapid phosphorylation of p42/44 MAP kinase. We confirmed that pretreatment of the cells with 20 μM PD98059, a MEK (MAP kinase kinase) inhibitor, abolished the 2-AG-induced phosphorylation of p42/44 MAP kinase (data not shown). The observation that 2-AG induces rapid phosphorylation of p42/44 MAP kinase strongly suggested that the enzyme activity of p42/44 MAP kinase is elevated in 2-AG–stimulated cells. Next, we examined this in detail using Elk-1 fusion protein as an enzyme substrate. We confirmed that p42/44 MAP kinase activity was rapidly augmented in 2-AG–stimulated cells. The activation of p42/44 MAP kinase in 2-AG–stimulated cells proceeded in a time-dependent manner (Fig. 2). The highest enzyme activity was noted in cells stimulated with 2-AG for 2 min. The enzyme activity was augmented dose-dependently (Fig. 3); the highest activity was observed with 1–10 μM 2-AG.

Next, we examined the effect of SR144528, a CB2 receptor–specific antagonist, on 2-AG–induced activation of p42/44 MAP kinase. We found that pretreatment of cells with SR144528 abolished the 2-AG–induced rapid activation of p42/44 MAP kinase (Fig. 4). This observation clearly indicates that the response induced by 2-AG is mediated

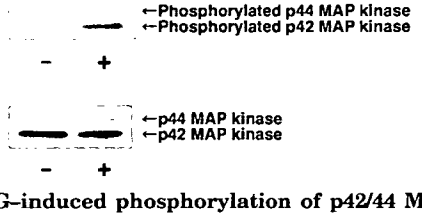

Fig. 1. **2-AG–induced phosphorylation of p42/44 MAP kinase in HL-60 cells.** HL-60 cells were incubated in the presence (+) or absence (−) of 2-AG (1 μM) for 3 min. Western blot analysis was performed as described in the text. The result is representative of four sets of independent experiments.

through the cannabinoid CB2 receptor. The effect of pertussis toxin (PTX)–treatment of the cells on 2-AG–induced activation of p42/44 MAP kinase was next examined. As shown in Fig. 5, PTX-treatment blocked the response induced by 2-AG (Fig. 5), indicating that G protein G_i/G_o is involved in 2-AG–induced activation of p42/44 MAP kinase.

Finally, we examined whether other cannabimimetic molecules exert a similar effect on p42/44 MAP kinase in this cell line. We found that CP55940, a synthetic cannabinoid, is capable of inducing the activation of p42/44 MAP kinase, like 2-AG (Fig. 6). We also found that anandamide induces the activation of p42/44 MAP kinase to some extent, although the magnitude of the stimulation was less pronounced than in the cases of 2-AG and CP55940 (Fig. 6).

It is clear from the above results that 2-AG, an endogenous cannabinoid receptor ligand, induces rapid phosphorylation and activation of p42/44 MAP kinase in HL-60 cells. To our knowledge, this is the first report of 2-AG–induced activation of p42/44 MAP kinase in mammalian cells. Previously, several investigators demonstrated that synthetic cannabinoids such as CP55940 and HU-210, and anandamide induce the activation of p42/44 MAP kinase (*14–23*). However, the effect of 2-AG was not examined in those previous studies. We have already demonstrated that 2-AG is the intrinsic natural ligand for the cannabinoid receptors (*6–9*); the finding in the present investigation is, therefore, of great interest.

The activation of p42/44 MAP kinase in HL-60 cells by 2-AG takes place in a cannabinoid CB2 receptor–dependent and G_i/G_o-dependent manner (Figs. 4 and 5). Previously, we found that 2-AG induces Ca^{2+} transients in HL-60 cells through a cannabinoid CB2 receptor–dependent and G_i/G_o-

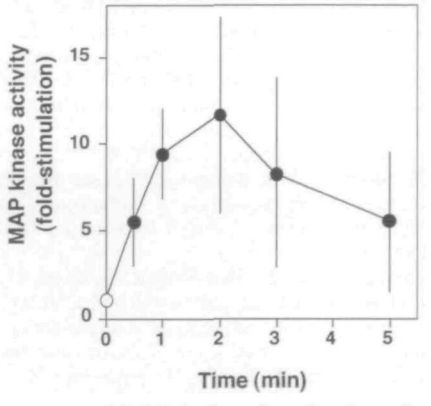

Fig. 2. **Time-course of 2-AG–induced activation of p42/44 MAP kinase in HL-60 cells.** HL-60 cells were stimulated with 2-AG (1 μM) for the indicated periods. The enzyme activity was determined using Elk-1 fusion protein as a substrate as described in the text. The value for the unstimulated control is set at 1.0. The data are the means ± SD for three separate experiments, each performed in duplicate.

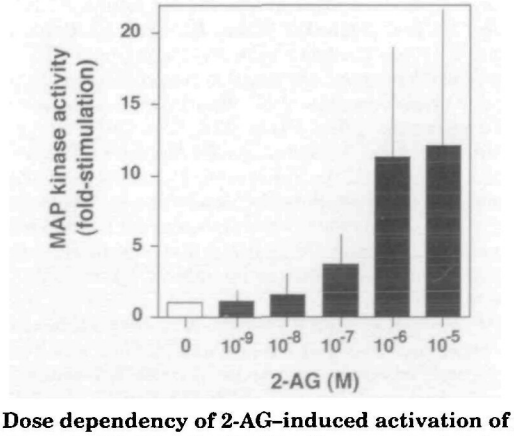

Fig. 3. **Dose dependency of 2-AG–induced activation of p42/44 MAP kinase in HL-60 cells.** HL-60 cells were stimulated with various concentrations of 2-AG for 2 min. The enzyme activity was determined as described in the text. The value for the control (DMSO alone) is set at 1.0. The data are the means ± SD for three separate experiments, each performed in duplicate.

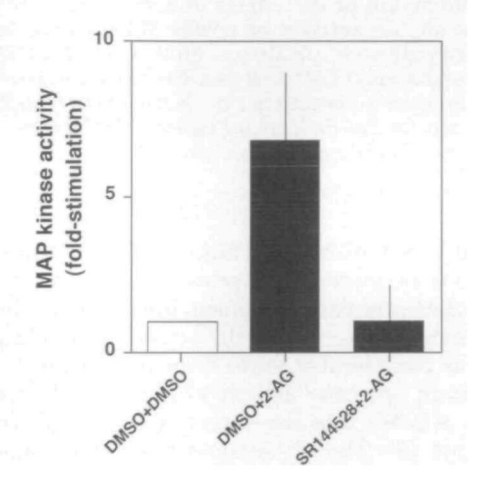

Fig. 4. **Effect of SR144528 on 2-AG–induced activation of p42/44 MAP kinase in HL-60 cells.** HL-60 cells were treated with SR144528 (1 μM) or DMSO for 1 min. Then, the cells were challenged with 2-AG (1 μM) for 2 min. The enzyme activity was determined as described in the text. The value for the control (DMSO alone) is set at 1.0. The data are the means ± SD for three separate experiments, each performed in duplicate.

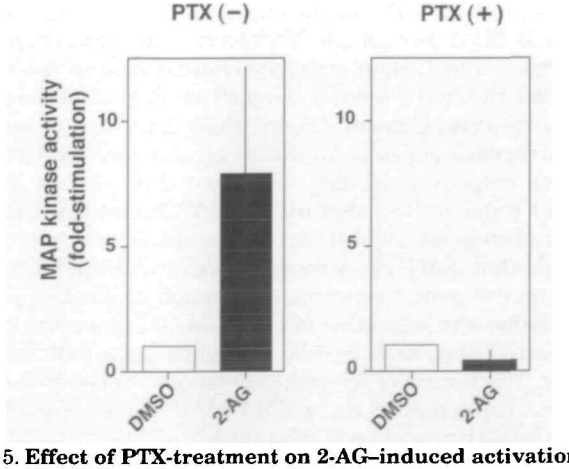

Fig. 5. **Effect of PTX-treatment on 2-AG–induced activation of p42/44 MAP kinase in HL-60 cells.** HL-60 cells were treated with PTX (100 ng/ml) for 16 h. Then, the cells were challenged with 2-AG (1 μM) for 2 min. The enzyme activity was determined as described in the text. The value for the control (DMSO alone) is set at 1.0. The data are the means ± SD for three separate experiments, each performed in duplicate.

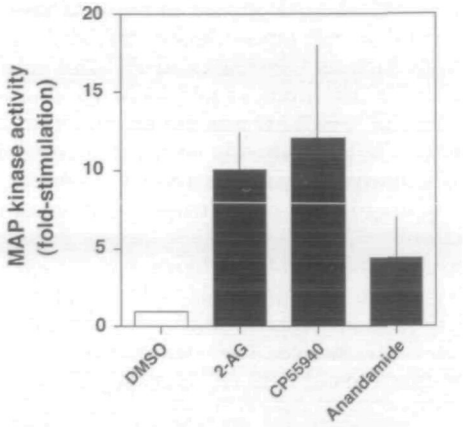

Fig. 6. **Comparison of the effects of several cannabimimetic molecules on the activity of p42/44 MAP kinase in HL-60 cells.** HL-60 cells were stimulated with 2-AG (1 μM), CP55940 (1 μM), or anandamide (1 μM) for 2 min. The enzyme activity was determined as described in the text. The value for the control (DMSO alone) is set at 1.0. The data are the means ± SD for three separate experiments, each performed in duplicate.

dependent mechanism (*9*). Thus, the signal transduction mechanisms of these two types of cellular responses induced by 2-AG are partly common. Interestingly, the capacity of anandamide to induce the activation of p42/44 MAP kinase was considerably lower than that of 2-AG (Fig. 6). Similar weak agonistic activity of anandamide, compared with that of 2-AG, was also observed in our Ca^{2+}-transient experiments (*9*). The observation that anandamide possesses only weak agonistic activity, in this study and in the previous study (*9*), is consistent with the idea that anandamide is not the natural ligand for the cannabinoid receptors (*24*).

What is the physiological meaning of 2-AG–induced activation of p42/44 MAP kinase? p42/44 MAP kinase is one of the key enzymes in intracellular signal transduction in diverse cell types. p42/44 MAP kinase phosphorylates transcription factors in the nucleus, thereby regulating gene transcription (*25*). The major nuclear target of p42/44 MAP kinase is Elk-1 protein (*26–28*). The reaction product, phosphorylated Elk-1, then activates transcription of the c-*fos* promoter through a ternary complex assembled on the c-*fos* serum response element (*26–28*). Such activation of immediate response genes is known to be essential for various cellular responses. In this study, we showed that 2-AG induces rapid activation of p42/44 MAP kinase using Elk-1 fusion protein as a substrate of the kinase reaction; it is possible that 2-AG, like various growth factors, induces the activation of gene transcription, through sequential phosphorylation and activation of p42/44 MAP kinase and transcription factors such as Elk-1, to elicit some cellular response. The nature of the cellular response linked to 2-AG–induced activation of the p42/44 MAP kinase cascade is under investigation in our laboratory.

Five years have passed since the identification of 2-AG as an endogenous cannabinoid receptor ligand. Evidence is gradually accumulating, through previous studies (*1–13*, *24*, *29*, *30*) and the present study (Figs. 1–6), that 2-AG possesses a variety of potent biological activities. It may act as an important mediator molecule in various mammalian tissues where the cannabinoid receptors are expressed. However, the available information concerning 2-AG is still limited compared with that on other structurally related lipid mediators such as lysophosphatidic acids (*24*). Further detailed studies are essential for a full understanding of the physiological and pathophysiological roles of 2-AG in mammalian tissues under various conditions.

During the preparation of this manuscript, Rueda *et al.* (*31*) reported that 2-AG induces the activation of c-Jun N-terminal kinase in CHO cells stably transfected with the CB1 receptor cDNA.

REFERENCES

1. Mechoulam, R., Devane, W.A., and Glaser, R. (1992) Cannabinoid geometry and biological activity in *Marijuana/Cannabinoids: Neurobiology and Neurophysiology* (Murphy, L. and Bartke, A., eds.), pp. 1–33, CRC Press, Boca Raton, FL
2. Sugiura, T., Kondo, S., Sukagawa, A., Nakane, S., Shinoda, A., Itoh, K., Yamashita, A., and Waku, K. (1995) 2-Arachidonoylglycerol: a possible endogenous cannabinoid receptor ligand in brain. *Biochem. Biophys. Res. Commun.* **215**, 89–97
3. Mechoulam, R., Ben-Shabat, S., Hanus, L., Ligumsky, M., Kaminski, N.E., Schatz, A.R., Gopher, A., Almog, S., Martin, B.R., Compton, D.R., Pertwee, R.G., Griffin, G., Bayewitch, M., Barg, J., and Vogel, Z. (1995) Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. *Biochem. Pharmacol.* **50**, 83–90
4. Varga, K., Wagner, J.A., Bridgen, D.T., and Kunos, G. (1998) Platelet- and macrophage-derived endogenous cannabinoids are involved in endotoxin-induced hypotension. *FASEB J.* **12**, 1035–1044
5. Mechoulam, R., Fride, E., Ben-Shabat, S., Meiri, U., and Horowitz, M. (1998) Carbachol, an acetylcholine receptor agonist, enhances production in rat aorta of 2-arachidonoyl glycerol, a hypotensive endocannabinoid. *Eur. J. Pharmacol.* **362**, R1–3
6. Sugiura, T., Kodaka, T., Kondo, S., Tonegawa, T., Nakane, S., Kishimoto, S., Yamashita, A., and Waku, K. (1996) 2-Arachidonoylglycerol, a putative endogenous cannabinoid receptor ligand, induces rapid, transient elevation of intracellular free Ca^{2+} in neuroblastoma x glioma hybrid NG108-15 cells. *Biochem. Biophys. Res. Commun.* **229**, 58–64
7. Sugiura, T., Kodaka, T., Kondo, S., Nakane, S., Kondo, H., Waku, K., Ishima, Y., Watanabe, K., and Yamamoto, I. (1997) Is the cannabinoid CB1 receptor a 2-arachidonoylglycerol receptor? Structural requirements for triggering a Ca^{2+} transient in NG108-15 cells. *J. Biochem.* **122**, 890–895
8. Sugiura, T., Kodaka, T., Nakane, S., Miyashita, T., Kondo, S., Suhara, Y., Takayama, H., Waku, K., Seki, C., Baba, N., and Ishima, Y. (1999) Evidence that the cannabinoid CB1 receptor is a 2-arachidonoylglycerol receptor. Structure-activity relationship of 2-arachidonoylglycerol, ether-linked analogues, and related compounds. *J. Biol. Chem.* **274**, 2794–2801
9. Sugiura, T., Kondo, S., Kishimoto, S., Miyashita, T., Nakane, S., Kodaka, T., Suhara, Y., Takayama, H., and Waku, K. (2000) Evidence that 2-arachidonoylglycerol but not *N*-palmitoylethanolamine or anandamide is the physiological ligand for the cannabinoid CB2 receptor. Comparison of the agonistic activities of various cannabinoid receptor ligands in HL-60 cells. *J. Biol. Chem.* **275**, 605–612
10. Lee, M., Yang, K.H., and Kaminski, N.E. (1995) Effects of putative cannabinoid receptor ligands, anandamide and 2-arachidonyl-glycerol, on immune function in B6C3F1 mouse splenocytes. *J. Pharmacol. Exp. Ther.* **275**, 529–536
11. Ouyang, Y., Hwang, S.G., Han, S.H., and Kaminski, N.E. (1998) Suppression of interleukin-2 by the putative endogenous cannabinoid 2-arachidonyl-glycerol is mediated through down-regulation of the nuclear factor of activated T cells. *Mol. Pharmacol.* **53**, 676–683
12. Stella, N., Schweitzer, P., and Piomelli, D. (1997) A second en-

dogenous cannabinoid that modulates long-term potentiation. *Nature* **388**, 773–778
13. Sugiura, T., Kodaka, T., Kondo, S., Tonegawa, T., Nakane, S., Kishimoto, S., Yamashita, A., and Waku, K. (1997) Inhibition by 2-arachidonoylglycerol, a novel type of possible neuromodulator, of the depolarization-induced increase in intracellular free calcium in neuroblastoma x glioma hybrid NG108-15 cells. *Biochem. Biophys. Res. Commun.* **233**, 207–210
14. Wartmann, M., Campbell, D., Subramanian, A., Burstein, S.H., and Davis, R.J. (1995) The MAP kinase signal transduction pathway is activated by the endogenous cannabinoid anandamide. *FEBS Lett.* **359**, 133–136
15. Bouaboula, M., Poinot-Chazel, C., Bourrie, B., Canat, X., Calandra, B., Rinaldi-Carmona, M., Le Fur, G., and Casellas, P. (1995) Activation of mitogen-activated protein kinases by stimulation of the central cannabinoid receptor CB1. *Biochem. J.* **312**, 637–641
16. Bouaboula, M., Poinot-Chazel, C., Marchand, J., Canat, X., Bourrie, B., Rinaldi-Carmona, M., Calandra, B., Le Fur, G., and Casellas, P. (1996) Signaling pathway associated with stimulation of CB2 peripheral cannabinoid receptor. Involvement of both mitogen-activated protein kinase and induction of Krox-24 expression. *Eur. J. Biochem.* **237**, 704–711
17. Bouaboula, M., Perrachon, S., Milligan, L., Canat, X., Rinaldi-Carmona, M., Portier, M., Barth, F., Calandra, B., Pecceu, F., Lupker, J., Maffrand, J.P., Le Fur, G., and Casellas, P. (1997) A selective inverse agonist for central cannabinoid receptor inhibits mitogen-activated protein kinase activation stimulated by insulin or insulin-like growth factor 1. Evidence for a new model of receptor/ligand interactions. *J. Biol. Chem.* **272**, 22330–22339
18. Derocq, J.M., Bouaboula, M., Marchand, J., Rinaldi-Carmona, M., Segui, M., and Casellas, P. (1998) The endogenous cannabinoid anandamide is a lipid messenger activating cell growth via a cannabinoid receptor-independent pathway in hematopoietic cell lines. *FEBS Lett.* **425**, 419–425
19. Rinaldi-Carmona, M., Le Duigou, A., Oustric, D., Barth, F., Bouaboula, M., Carayon, P., Casellas, P., and Le Fur, G. (1998) Modulation of CB1 cannabinoid receptor functions after a long-term exposure to agonist or inverse agonist in the Chinese hamster ovary cell expression system. *J. Pharmacol. Exp. Ther.* **287**, 1038–1047
20. Rinaldi-Carmona, M., Barth, F., Millan, J., Derocq, J.M., Casellas, P., Congy, C., Oustric, D., Sarran, M., Bouaboula, M., Calandra, B , Portier, M., Shire, D., Breliere, J.C., and Le Fur, G. (1998) SR 144528, the first potent and selective antagonist of the CB2 cannabinoid receptor. *J. Pharmacol. Exp. Ther.* **284**, 644–650
21. Bouaboula, M., Desnoyer, N., Carayon, P., Combes, T., and Casellas, P. (1999) Gi protein modulation induced by a selective inverse agonist for the peripheral cannabinoid receptor CB2: implication for intracellular signalization cross-regulation. *Mol. Pharmacol.* **55**, 473–480
22. Bouaboula, M., Bianchini, L., McKenzie, F.R., Pouyssegur, J., and Casellas, P. (1999) Cannabinoid receptor CB1 activates the Na^+/H^+ exchanger NHE-1 isoform via G_i-mediated mitogen activated protein kinase signaling transduction pathways. *FEBS Lett.* **449**, 61–65
23. Liu, J., Gao, B., Mirshahi, F., Sanyal, A.J., Khanolkar, A.D., Makriyannis, A., and Kunos, G. (2000) Functional CB1 cannabinoid receptors in human vascular endothelial cells. *Biochem. J.* **346**, 835–840
24. Sugiura, T. and Waku, K. (2000) 2-Arachidonoylglycerol and the cannabinoid receptors. *Chem. Phys. Lipids* **108**, 89–106
25. Seger, R. and Krebs, E.G. (1995) The MAPK signaling cascade. *FASEB J.* **9**, 726–735
26. Gille, H., Sharrocks, A.D., and Shaw, P.E. (1992) Phosphorylation of transcription factor $p62^{TCF}$ by MAP kinase stimulates ternary complex formation at c-*fos* promoter. *Nature* **358**, 414–417
27. Marais, R., Wynne, J., and Treisman, R. (1993) The SRF accessory protein Elk-1 contains a growth factor-regulated transcriptional activation domain. *Cell* **73**, 381–393
28. Gille, H., Kortenjann, M., Thomae, O., Moomaw, C., Slaughter, C., Cobb, M.H., and Shaw, P.E. (1995) ERK phosphorylation potentiates Elk-1-mediated ternary complex formation and transactivation. *EMBO J.* **14**, 951–962
29. Di Marzo, V., Melck, D., Bisogno, T., and De Petrocellis, L. (1998) Endocannabinoids: endogenous cannabinoid receptor ligands with neuromodulatory action. *Trends Neurosci.* **21**, 521–528
30. Piomelli, D., Beltramo, M., Giuffrida, A., and Stella, N. (1998) Endogenous cannabinoid signaling. *Neurobiol. Dis.* **5**, 462–473
31. Rueda, D., Galve-Roperh, I., Haro, A., and Guzman, M. (2000) The CB(1) cannabinoid receptor is coupled to the activation of c-Jun N-terminal kinase. *Mol. Pharmacol.* **58**, 814–820