2-Arachidonylglycerol Stimulates Activator Protein-1-dependent Transcriptional Activity and Enhances Epidermal Growth Factor-induced Cell Transformation in JB6 P+ Cells*

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2-Arachidonylglycerol (2-AG) is the most abundant endocannabinoid, and it plays a critical role in cannabinoid receptor-mediated cell signaling. Although 2-AG was shown to induce ERK activation via the cannabinoid receptor 1 (CB1), only a nonspecific CB receptor agonist and antagonist was used in those studies. Whether cannabinoid receptor 2 (CB2) is involved in 2-AG-induced ERK activation is still unclear. Moreover, whether 2-AG is involved in mediation of AP-1 activity and cell transformation is also not known. In the present study, we show that 2-AG stimulates AP-1-dependent transcriptional activity and enhances epidermal growth factor-induced cell transformation in mouse epidermal JB6 P+ Cl41 cells. Using JB6 P+ Cl41 cells, stably transfected with an AP-1 luciferase reporter, we found that 10 μM 2-AG induced up to a 3-fold stimulation of AP-1 transcriptional activity. The AP-1 stimulation appeared to be mediated by ERK but not JNK or p38 kinase.

2-Arachidonylglycerol and cannabinoids were also shown to stimulate mitogen-activated protein (MAP) kinases, including extracellular signal-regulated kinases (ERKs) (10–12), c-Jun N-terminal kinases (JNKs), and p38 kinases (12–15) and to stimulate B-cell (16) and splenocyte (17) proliferation under certain conditions. Cannabinoids were also shown to stimulate sequence-specific AP-1 DNA-binding activity (18). Also, cannabinoid receptor ligands may have opposite effects depending on concentration (19) and other experimental conditions (11, 13). The expression of cytokine mRNAs was also shown to be differentially affected by cannabinoids depending on cell line and the cytokine tested (20). Hence, cell signaling induced by cannabinoid receptor ligands needs additional and extensive investigation.

The most thoroughly investigated cannabinoid receptor ligands are anandamide and 2-arachidonylglycerol (2-AG). Both of these ligands occur in trace amounts in virtually all vertebrate cells and tissues (21). Although both anandamide and 2-AG are ligands for cannabinoid receptors, significant differences exist between these two agonists. Recent work has clearly shown that 2-AG is a full agonist for both cannabinoid 1 (CB1; 22–24) and cannabinoid 2 (CB2; 25, 26) receptors, whereas anandamide is only a partial agonist for CB1 and CB2.

2-Arachidonylglycerol is believed to induce cell signaling through the cannabinoid receptor-mediated cell signaling. 2-Arachidonylglycerol and cannabinoids were also shown to stimulate mitogen-activated protein (MAP) kinases, including extracellular signal-regulated kinases (ERKs) (10–12), c-Jun N-terminal kinases (JNKs), and p38 kinases (12–15) and to stimulate B-cell (16) and splenocyte (17) proliferation under certain conditions. Cannabinoids were also shown to stimulate sequence-specific AP-1 DNA-binding activity (18). Also, cannabinoid receptor ligands may have opposite effects depending on concentration (19) and other experimental conditions (11, 13). The expression of cytokine mRNAs was also shown to be differentially affected by cannabinoids depending on cell line and the cytokine tested (20). Hence, cell signaling induced by cannabinoid receptor ligands needs additional and extensive investigation.

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Porcella et al. (18) reported that 99-THC, a major psychoactive cannabinoid, up-regulated the mRNA levels of immediate-early genes in the rat brain. 99-THC was shown to increase sequence-specific AP-1 DNA-binding activity by acting on the cannabinoid receptors (18). We reported that N-acetylenolamines and anandamide stimulate cannabinoid-receptor-independent ERK phosphorylation and AP-1-dependent transcriptional activity up to 2-fold in mouse epidermal JB6 cells (27). Whether 2-AG directly regulates AP-1-dependent transcriptional activity and is involved in cell transformation is as yet unknown. We addressed this question by studying signal transduction initiated by 2-AG in mouse epidermal JB6 P+ C141 cells, which provide a model system used extensively to study signal transduction and neoplastic transformation (28–31). The signaling pathways resulting in activation of AP-1 are well characterized in these cells, and JB6 cells were shown to synthesize both anandamide (27) and 2-AG (32) and also express cannabinoid receptors (18). We reported that 2-AG and 9-THC, a major psychoactive cannabinoid, up-regulated the mRNA levels of immediate-early genes in the rat brain.

The primers used for PCR verification of CB2 from tail DNA and cellular DNA were: primer 1 (5'-ATATGCTTTAGTTGGTGTCAGCCTCTC-3'), primer 2 (5'-TAAGGCAGTTCGAGATCAGGCTT-3'), and primer 3 (5'-GCCCTTCCTGTTTTCACATTGCCTCCT-3'). The PCR amplification was done at 95 °C for 1 min, 60 °C for 1 min, 72 °C for 2 min, and was carried out for 30 cycles using Taq polymerase. For CB1/2+/+ cells and mice, we obtained a 1100-bp band, and for CB1/2−/− cells and mice, we obtained a 850-bp band (data not shown). Therefore the genotype for the CB1/2−/− cells and mice is correct.

**Cell Transformation**—For stable transfections, according to the protocol from Invitrogen (Carlsbad, CA), we transfected CB1/2−/− cells with CB1 or CB2 expression constructs using the Lipofectamine 2000 reagent (Invitrogen) as specified by the supplier. All the transfected CB1/2−/− cells were selected for 2 weeks in medium containing 400 μg/mL of G418 after which time the G418 concentration was decreased to 200 μg/mL and maintained.

**Establishing Dominant Negative (DN)-Fyn and Small Interfering (si)-RNA Fyn Expressing JB6 Cl41 Cells**—The K2990 Fyn plasmid was provided by Dr. Moses V. Chao from the New York University Medical Center (First Avenue, New York). Using these plasmids, we established stable transfections according to the protocol from Invitrogen. All the transfected JB6 cells were selected for 2 weeks in media containing 400 μg/mL of G418 after which time the G418 concentration was decreased to 200 μg/mL and maintained. siRNA Fyn JB6 cells were transfected with siRNA previously described (33). Briefly, we disrupted Fyn expression by the siRNA method. The sense oligonucleotide of Fyn used for siRNA was 5'-TTTGCGACTGGAGAAGATTGGTTCAAGAGAC-3', and the antisense was 5'-TTTGCGACTGGAGAAGATTGGTTCAAGAGAC-3'. The liqated pair of oligonucleotides was inserted into the mU6 vector. The oligonucleotide synthesis and sequencing of the inserts were performed by Sigma. The plasmids siRNA-Fyn-mU6pro was stably transfected into JB6 Cl41 cells using the Lipofectamine 2000 reagent.

**Cell Culture**—JB6 P+ mouse epidermal cells (C141) and AP-1 luciferase reporter (C141 AP-1 mass 1) stable transfectants were cultured in monolayers at 37 °C in MEM containing 5% heat-inactivated FBS, 2 mM l-glutamine, and 25 μg/mL gentamicin in a humidified atmosphere containing 5% CO2.

**AP-1 Activity Assay**—Confluent monolayers of JB6 P+ C141 cells (5 × 106), stably transfected with the luciferase reporter driven by AP-1, were suspended in 200 μL of 5% FBS/MEM and added into each well of a 96-well plate. Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO2. Twenty-four to forty-eight hours later, cells were washed by culturing in 100 μL of 0.1% FBS/MEM for 24 h before treatment with final concentrations of 500 nM 2-AG for 6 h. The cells were extracted with lysis buffer and luciferase activity was measured using the Promega Luciferase Assay System (Promega, Madison, WI) and the Luminoskan Ascent (ThermoElectron Corp., Helsinki, Finland). The results are expressed as relative AP-1 activity compared with an untreated control value of 1.

**Fyn Kinase Activity Assay**—The assay for ERK activity was carried out as described in the protocol provided by Cell Signaling Technology (Beverly, MA). In brief, JB6 P+ cells (wild type Fyn) and DN-Fyn cells were starved for 48 h in 0.1% FBS/MEM and then treated with 10 μM 2-AG for 30 min. The cells were then washed once with ice-cold phosphate-buffered saline and disrupted in 300 μL of cell lysis buffer. The lysates were sonicated and centrifuged. Endogenous ERKs were immunoprecipitated from the supernatant fraction containing 300 μg of protein by incubating with the specific phospho-specific ERK antibody (Th202/Tyr204) overnight at 4 °C, followed by incubation with protein A/G plus-agarose beads for another 4 h. The beads were washed twice with 50 μL of PBS (pH 7.5) and twice with 500 μL of kinase buffer (50 mM Tris, pH 7.5, 5 mM β-glycerophosphate, 2 mM dithiothreitol, 0.1 mM NaVO3, and 10 mM MgCl2). For determination of ERK-induced phosphorylation of Elk-1, the kinase reactions were performed at 30 °C for 30 min in 50 μL of the kinase buffer containing the immunoprecipitates and 200 μM ATP with 2 μg of Elk-1 fusion protein as the substrate. Phosphorylation of Elk-1 was then analyzed by Western blotting using a chemiluminescent detection system and specific antibodies against phosphorylation of Elk-1 at serine 383.

**Fyn Kinase Assay**—Analysis of Fyn kinase activity was carried out as described in the protocol provided by Upstate (Charlottesville, VA). In brief, the cells were treated for the indicated times with 10 μM 2-AG and different doses of PP2 or its vehicle, MeSO (<0.1%), which was used as a negative control. The procedure was essentially the same as that for assay of ERK activity except that the immunoprecipitates were com-
bined with 10 μl of [32P]ATP and 2.5 μl of the Src substrate peptide. Fyn kinase activity was then determined by scintillation counter.

**Western Blotting—ERK, JNK, and p38 kinase phosphorylation were determined by immunoblotting with phospho-specific antibodies against ERK1/2 (Thr202/Tyr204), JNK1/2 (Thr183/Tyr185), and p38 kinases (Thr180/Tyr182), respectively. Antibodies bound to proteins were detected by chemifluorescence (ECF substrate, Amersham Biosciences) using the Storm 840 Imaging System (Amersham Biosciences). Some membranes were stripped (7 M guanidine hydrochloride, 50 mM glycine (pH 10.8), 0.05 mM EDTA, 0.1 mM KCl, 20 mM 2-mercaptoethanol), and re-probed.

**Anchorage-independent Transformation Assay—**The role of 2-AG in EGF-promoted cell transformation was investigated in JB6 C141 cells (33). In brief, 8 × 10⁵/ml cells were exposed to EGF (0–0.1 ng/ml) with or without 2-AG (1–10 μM) in 1 ml of 0.3% basal medium Eagle’s agar containing 10% FBS. The cultures were maintained at 37 °C in a 5% CO₂ incubator for 10 days, and the cell colonies were scored as described previously (29). The effect of 2-AG and EGF on JB6 C141 cell transformation is presented as colony number per 8,000 seeded JB6 C141 cells in soft agar.

**AP-1 DNA Binding Study—**Nuclear protein extracts were prepared from cells, as described previously (29). Briefly, JB6 P⁺ C141 cells were cultured in 10-cm dishes and starved in 0.1% FBS/MEM at 37 °C in a 5% CO₂ incubator. After 24 h of starvation, the cells were exposed to different concentrations of EGF or 2-AG or EGF and 2-AG for 12 h. The cells were then harvested and disrupted in 500 μl of lysis buffer A (50 mM KCl, 0.5% Nonidet P-40, 100 μM dithiothreitol, 25 mM HEPES, pH 7.8, 10 μg/ml leupeptin, 25 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride). After a 1-min centrifugation (16,000 × g at 4 °C), the pellets containing the nuclei were washed once with 500 μl of Buffer B (Buffer A without Nonidet P-40). The pellets were then resuspended in 100 μl of extraction buffer (Buffer B, but with 50 mM KCl and 10% glycerol) and strongly shaken at 4 °C for 30 min. After a 10-min centrifugation (16,000 × g at 4 °C), the supernatant solutions were moved into fresh tubes and stored at −70 °C until analysis. The DNA binding reaction was incubated at room temperature for 20 min in a mixture containing 5 μg of nuclear protein, 1 μg of poly(dI:dC), and 15,000 cpm of a 5'-CGCTT-GATGAGTCAGCCGGAA-3'). The samples were separated on a 5% polyacrylamide gel, and the gels were analyzed using the Storm 840 phosphorimaging system (Amersham Biosciences).

**RESULTS**

**2-AG-stimulated ERK Phosphorylation and AP-1 Transcriptional Activity—**MAP kinases are important elements of cell signaling cascades that regulate cell growth, differentiation, and tumorigenic transformation. When cells were treated with 2-AG, this endocannabinoid was found to stimulate ERK phosphorylation in a dose-dependent manner in JB6 P⁺ cells (Fig. 1A) but had no effect on phosphorylation of JNKs or p38 kinase (Fig. 1A). Because ERKs are upstream of and known to activate the AP-1 transcription factor (36), 2-AG-induced ERK activation may also result in enhanced AP-1-dependent transcriptional activity. Indeed, we found that 10 μM 2-AG significantly stimulated AP-1-dependent transcription activity (Fig. 1B, *p < 0.05).

**2-AG-stimulated ERK Phosphorylation and AP-1 Transcriptional Activity Were Blocked by PD98059—**To determine whether 2-AG stimulation of AP-1 transcriptional activity is mediated directly by ERK phosphorylation, PD98059, a specific inhibitor of MEK1, which is a kinase upstream from ERKs, was used. Results indicate that PD98059 almost completely blocked 2-AG-induced ERK phosphorylation (Fig. 2A) and significantly inhibited AP-1-dependent transcriptional activity (Fig. 2B, *p < 0.05) in JB6 cells.

**2-AG-stimulated ERK Phosphorylation Occurs Through CB1 and CB2—**2-AG was reported to stimulate ERK phosphorylation in other cells (10–12, 34, 35). However, direct evidence of whether this induction occurs through CB1 and CB2 is lacking. We therefore used CB1⁻/⁻ and CB2⁻/⁻ mice to address this question. CB1⁻/⁻ and CB2⁻/⁻ MEFs were obtained and verified to be correct as described under “Materials and Methods.” Treatment of cells with 10 μM 2-AG for the indicated time (Fig. 3A) or dose (Fig. 3B) resulted in a time- and dose-dependent increase in ERK phosphorylation in CB1/2⁺/⁺ cells, but not in CB1/2⁻/⁻ cells (Fig. 3, A and B). However, if CB1⁻/⁻ and CB2⁻/⁻ cells were treated with 10 ng/ml of TPA, a phorbol ester known to induce ERK phosphorylation, for the indicated times, ERK phosphorylation increased in a time-dependent manner not only in CB1/2⁺/⁺ cells but also in CB1/2⁻/⁻ cells (Fig. 3C), suggesting that ERK response to TPA occurs independent of the CB receptor. On the other hand, 2-AG induction of ERK phosphorylation occurs through the CB1/2 receptors.

**2-AG-induced ERK Phosphorylation Was Not Blocked by the PI-3K Inhibitor LY294002—**Cannabinoids were reported to increase ERK activity through recruitment of PI-3K (36, 37). Therefore, we used LY294002, a specific inhibitor of PI-3K, to...
study the role of PI-3K in 2-AG-induced ERK phosphorylation in JB6 P" cells. However, treatment of cells with 25–50 μM LY294002 for 30 min had no significant effect on 2-AG-induced ERK phosphorylation (Fig. 5), suggesting that 2-AG-induced ERK phosphorylation in JB6 P" cells does not occur through the PI-3K pathway.

2-AG-induced ERK Phosphorylation and Activity Were Blocked by PP2 or DN-Fyn or siRNA-Fyn—The Src family kinase, Fyn, was reported to play a critical role in the activation of ERKs by CB1 receptors based on the finding that this activation was absent in Fyn mutant mice (34). Because PP2 is an inhibitor of Src family kinases, including Fyn, we treated JB6 P" cells with PP2 for 45 min, followed by treatment with 10 μM 2-AG for 30 min. Results showed that 0.5–1.0 μM PP2 almost completely blocked the 2-AG-induced ERK phosphorylation (Fig. 6A). To further determine whether 2-AG-induced ERK phosphorylation is mediated through Fyn, we employed JB6 wild-type-Fyn and DN-Fyn cells and cells transfected with siRNA-Fyn. Results showed that 2-AG-induced ERK phosphorylation in JB6 wild-type-Fyn cells, but 2-AG-induced ERK phosphorylation was almost completely suppressed in DN-Fyn cells (Fig. 6B) and in siRNA-Fyn cells (Fig. 6C). Additional results also indicate that 2-AG-induced ERK activity was attenuated by PP2 or DN-FYN (Fig. 7).

2-AG-induced Fyn Kinase Activity Was Blocked by PP2 or DN-Fyn—We also studied the effect of PP2 and DN-Fyn on Fyn kinase activity. We found that 1.0 μM PP2 markedly decreased 2-AG-induced Fyn kinase activity, and 2-AG-induced Fyn kinase activity was almost totally blocked in DN-Fyn cells (Fig. 8).

2-AG-stimulated Fyn Kinase Activity Is Mediated through CB1/2—To further determine whether 2-AG-stimulated Fyn kinase activity is mediated through CB1/2, we treated CB1/2"/+ or CB1/2"−/− cells with 1 μM PP2 for 45 min followed by treatment with 10 μM 2-AG for 30 min and then measured Fyn kinase activity. The results indicated that 2-AG significantly increased Fyn kinase activity in CB1/2"/+ cells and that 1.0 μM PP2 almost totally blocked 2-AG-stimulated Fyn kinase activity in these cells (Fig. 9, lanes 6–9), further confirming that 2-AG-stimulated Fyn kinase activity is mediated through CB1/2.

2-AG Enhancement of EGF-induced JB6 P" Cell Transformation—Because our previous results and other studies demonstrated that induction of AP-1 activity is required for cell transformation, we tested whether 2-AG could induce transformation or promote EGF-induced transformation. The results showed that 2-AG could slightly induce JB6 P" cell transformation (Fig. 10A, lane 4). Interestingly, a low concentration
2-AG Enhances EGF-induced AP-1 DNA Binding—To investigate whether 2-AG affected basal AP-1 DNA binding or EGF-induced AP-1 DNA binding in JB6 cells, we exposed JB6 P+ cells to 2-AG, EGF, or EGF plus 2-AG. The results showed that 2-AG induced AP-1 DNA binding (Fig. 11, lane 7) and enhanced EGF-induced AP-1 DNA binding (Fig. 11, lanes 8 and 9).

2-AG Enhances EGF-induced ERK Phosphorylation—Either 2-AG or EGF can induce ERK phosphorylation but whether 2-AG can affect EGF-induced ERK phosphorylation is as yet unknown. We analyzed the effect of 2-AG on EGF-induced ERK phosphorylation. As shown in Fig. 12, 2-AG not only induced ERK phosphorylation, but also enhanced EGF-induced ERK phosphorylation.
DISCUSSION

Cannabis occurs naturally in the dried flowering or fruiting tops of the Cannabis sativa plant. Cannabis is most often consumed by smoking marijuana. Recent studies suggested an association of marijuana smoking with head and neck cancers and oral lesions (38). Cannabinoids are the active compounds extracted from cannabis and endocannabinoids are defined as endogenously generated messenger molecules that bind to and activate cannabinoid receptors (21). Cannabinoid receptors are therefore be considered a new autacoid for regulating cellular function. Thus establishing the details of 2-AG-induced signaling is important to better understand the physiological significance of its production.

Recently, Carrier et al. (11) reported that cultured rat microglial cells synthesize the endocannabinoid 2-arachidonoylglycerol, which increases proliferation via a CB2 receptor ERK-dependent mechanism. Jorda et al. (12, 35) reported that CB2 may be involved in the induction of leukemic transformation. CB2 is aberrantly expressed in a high percentage of human acute myeloid leukemias. Aberrant expression of CB2 in hematopoietic precursor cells resulted in distinct effects depending on the ligand used. CB2-expressing myeloid precursors migrate upon stimulation by the endocannabinoid 2-arachidonoylglycerol and are blocked in neutrophilic differentiation upon exposure to another ligand, CP55940. Both effects depend on the ligand used. CB2-expressing leukemic cells respond to cannabinoid activation of AP-1 can therefore be considered a new autacoid for regulating cellular function. Thus establishing the details of 2-AG-induced signaling is important to better understand the physiological significance of its production.

This ligand is present in many cells at significantly higher levels than anandamide, the other known endocannabinoid (42, 43). Also, 2-AG was shown to be a full agonist for both CB1 and CB2 receptors, whereas anandamide serves only as a partial agonist for these receptors (21). Thus 2-AG is likely to play a critical role in cannabinoid receptor-mediated cell signaling. Importantly, 2-AG but not anandamide, was shown to be generated and released within seconds upon stimulation of cultured macrophages with platelet-activating factor (42) and can therefore be considered a new autacoid for regulating cellular function. Thus establishing the details of 2-AG-induced signaling is important to better understand the physiological significance of its production.
phosphorylation and AP-1-dependent transcriptional activity in mouse epidermal JB6 cells. Watts et al. (44) showed that expression of dominant negative ERK2 inhibits AP-1 transcription and neoplastic transformation. Huang et al. (45) demonstrated that the lack of AP-1 activation and cell transformation responses to TPA or EGF in F- cells appears to be because of a low level of ERKs in these cells. Although 9-THC increases sequence-specific AP-1 DNA-binding activity (18), whether 2-AG stimulation leads to increased AP-1 activity in JB6 cells was not addressed. Our current results indicated that the activation of the ERK pathway by 2-AG resulted in a pronounced up-regulation of AP-1-dependent transcriptional activity (Fig. 1B). Moreover, the MEK inhibitor PD98059 totally blocked 2-AG-induced ERK phosphorylation and AP-1 activation (Fig. 2, A and B), indicating 2-AG can induce AP-1 activation through ERKs and therefore may have a novel role in carcinogenesis.

Although several studies indicated that 2-AG-induced ERK activity occurs through the cannabinoid receptors (10–12, 34, 35), these studies only used cannabinoid receptor inhibitors or MEK inhibitors to block 2-AG-induced ERK activity, and therefore direct in vivo evidence is lacking. Using stably transfected Chinese hamster ovary cells expressing human CB1, Bouaboula et al. (10) showed that cannabinoid treatment induces both ERK phosphorylation and activation in a time- and dose-dependent manner and also that these effects are inhibited by SR 141716A, a selective CB1 antagonist. The activation of ERKs is blocked by pertussis toxin (10). Carrierre et al. (11) reported that 2-AG induces ERK activity in cultured rat microglial cells and increases proliferation through a CB2 receptor ERK-dependent mechanism, the effects of which are blocked by the CB2 antagonist SR 144528. Derkinderen et al. (34) and Jorda et al. (12, 35) showed that the MEK inhibitor PD98059 blocks 2-AG-induced ERK activity. In the present study, we prepared and identified CB1/2+ and CB1/2− MEFs and then treated the cells with 2-AG. We found that ERK phosphorylation increased only in the CB1/2+ cells and was almost totally blocked in CB1/2− cells (Fig. 3C). These data provide strong evidence that 2-AG-induced ERK phosphorylation is mediated almost completely through the CB1/2 receptors. To distinguish between the involvement of CB1 and CB2 in 2-AG-induced ERK phosphorylation, we transfected a CB1 or CB2 plasmid into CB1/2− cells and checked the effect on 2-AG-induced ERK phosphorylation. The result indicates that both CB1 and CB2 take part in 2-AG-induced ERK phosphorylation (Fig. 4). This is the first direct evidence indicating that CB2 is involved in 2-AG-induced ERK phosphorylation.

Although 2-AG can induce ERK phosphorylation (10–12, 34, 35), the precise mechanism of the activation of ERKs occurring through the cannabinoid receptors in JB6 cells is unclear. Our studies revealed a role for Fyn in 2-AG-induced signaling in JB6 P+ cells. 2-AG is an endocannabinoid, and cannabinoids produce their effects by binding to specific plasma membrane G protein-coupled receptors (46). Src tyrosine kinase is a novel direct effector of G proteins (47), and many G protein-mediated physiologic functions are sensitive to tyrosine kinase inhibitors. Activation of many G protein-coupled receptors has been shown to increase the activity of the Src family tyrosine kinases (47). Our data showed that 2-AG induced ERK phosphorylation, which was blocked by the Src inhibitor PP2 (Figs. 6A and 7), supporting a role for Src family tyrosine kinases in 2-AG-induced ERK activity. Li et al. (48) reported that the activation of Fyn is coupled to the ERK pathway. Overexpression of knockdown-Fyn in SCC9 cells dramatically down-regulated the activity of c-Raf and ERK and MMP-3 promoter activity. In this study, we used DN-Fyn and siRNA-Fyn to study the role of Fyn in 2-AG-induced ERK activity. We found that 2-AG-induced ERK activity was almost completely blocked by DN-Fyn and siRNA-Fyn (Fig. 6, B and C, and Fig. 7). In addition, 2-AG-induced Fyn kinase activity was also blocked by PP2 or DN-Fyn (Fig. 8), indicating an association between Fyn activation and stimulation of the ERKs-AP-1 signaling pathway in JB6 P+ cells. These results suggested that Fyn may play a very important role in 2-AG-stimulated ERK signaling and cancer development.

Studies in Chinese hamster ovary cells supported a role for PI-3K in CB1-ERKs coupling (10). Activation of ERKs by G-coupled receptors can be mediated through recruitment of PI-3K, independently of the inhibition of adenylcyclase (36). However, in hippocampal slices, the cannabinoid-induced activation of ERKs was insensitive to the PI-3K inhibitor LY294002, strongly arguing against a role for the PI-3K pathway in this effect (34). Our results indicated that LY294002 had no significant effect on 2-AG-induced ERK phosphorylation (Fig. 5), indicating that 2-AG-induced ERK phosphorylation in JB6 P+ cells was independent of PI-3K pathway activation.

2-AG can induce AP-1 transcriptional activity, but whether it is involved in cell transformation is unclear. Our data demonstrated that 2-AG enhances EGF-induced cell transformation (Fig. 10A), and AM251 and PD98059 almost totally blocked the enhancement (Fig. 10B). We further showed that this may be because of the ability of 2-AG to enhance EGF-induced AP-1 DNA binding (Fig. 11) and ERK phosphorylation (Fig. 12).

We hypothesize that 2-AG binds to both CB1 and CB2 receptors and that the stimulation of CB1 and CB2 leads to the recruitment of Fyn and activation of ERKs by Fyn (Fig. 13). Our data support this hypothesis, because Fyn kinase activity was blocked in CB1/2− cells. The present findings document for the first time a direct link between 2-AG stimulation and activation of the Fyn-ERK-AP-1 signaling pathway in JB6 CI41 cells. Thus in JB6 P+ cells, 2-AG appears to have a novel role in cell transformation and carcinogenesis in a signaling path-

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**Fig. 13. CB1/2, Fyn, ERK, but not JNK or p38 kinase, are involved in 2-AG-induced AP-1-dependent transcriptional activity in JB6 P+ cells.** CB1 and CB2 are at the beginning of multiple signal transduction pathways that are activated by 2-AG. Subsequently, Fyn contributes to ERK-mediated AP-1 signaling activation. The arrows or bars indicate activation or inhibition, respectively. The *p* indicates phosphorylation.
way involving the CB1/2 receptors and activation of Fyn, ERK, and AP-1.

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