0026-895X/03/6302-429–438$7.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 63:429–438, 2003

Vol. 63, No. 2
1898/0136101
Printed in U.S.A.

Anandamide Inhibits Nuclear Factor-κB Activation through a Cannabinoid Receptor-Independent Pathway

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Received June 5, 2002; accepted October 21, 2002

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

Anandamide (arachidonoylethanolamine, AEA), an endogenous agonist for both the cannabinoid CB_1 receptor and the vanilloid VR1 receptor, elicits neurobehavioral, anti-inflammatory, immunomodulatory, and proapoptotic effects. Because of the central role of nuclear factor-κB (NF-κB) in the inflammatory process and the immune response, we postulated that AEA might owe some of its effects to the suppression of NF-κB. This study shows that AEA inhibits tumor necrosis factor-α (TNFα)-induced NF-κB activation by direct inhibition of the IκB kinase (IKK)β and, to a lesser extent, the IKKα subunits of κB inhibitor (IκB) kinase complex, and that IKKs inhibition by AEA correlates with inhibition of IκBα degradation, NF-κB binding to DNA, and NF-κB-dependent transcription in TNFα-stimulated cells. AEA also prevents NF-κB-dependent reporter gene expression induced by mitogen-activated protein kinase kinase kinase and NF-κB-inducing kinase. The NF-κB inhibitory activity of AEA was independent of CB_1 and CB_2 activation in TNFα-stimulated 5.1 and A549 cell lines, which do not express vanilloid receptor 1, and was not mediated by hydrolytic products formed through the activity of the enzyme fatty acid amide hydrolase. Chemical modification markedly affected AEA inhibitory activity on NF-κB, suggesting rather narrow structure-activity relationships and the specific interaction with a molecular target. Substitution of the alkyl moiety with less saturated fatty acids generally reduced or abolished activity. However, replacement of the ethanolamine "head" with a vanillyl group led to potent inhibition of TNFα-induced NF-κB-dependent transcription. These findings provide new mechanistic insights into the anti-inflammatory and proapoptotic activities of AEA, and should foster the synthesis of improved analogs amenable to pharmaceutical development as anti-inflammatory agents.

Endocannabinoids are a class of lipid mediators found in several tissues and structurally based on a polyunsaturated fatty acid amide or ester motifs (Di Marzo et al., 1999). Anandamide (arachidonoylethanolamide, AEA) and 2-arachidonoylglicerol (2-AG) are the main endocannabinoids described to date. They act as mediators in the brain and in peripheral tissues mainly through the stimulation of brain (CB_1) and peripheral (CB_2) cannabinoid receptors. Although AEA preferentially binds to CB_1, 2-AG is equipotent at both receptor subtypes. AEA is produced by neurons and other cell types from the hydrolysis of the phospholipid precursor *N*-arachydonoylphosphatidylethanolamide, catalyzed by a Ca^{2+}-dependent phospholipase D (Di Marzo et al., 1994). Signal termination for AEA includes cellular reuptake by the AEA membrane transporter and hydrolysis by the fatty acid amide hydrolase (FAAH) (Di Marzo et al., 1994, 1999), a process that generates arachidonic acid and ethanolamine. Synaptic release of AEA is tightly regulated by depolarizing stimuli and glutamate receptor stimulation (Di Marzo et al., 1994). The degradative processes are also subject to regulation, and pharmacological inhibition of FAAH and AEA membrane transporter (Di Marzo et al., 1999; Boger et al., 2000) has been pursued as a way to increase the synaptic levels of AEA.

AEA can also interact with the vanilloid receptor type 1 (VR1) (Zygmunt et al., 1999; Smart et al., 2000). This ligand-gated cation channel is modulated allosterically by capsaicin and its analogs, and is mainly expressed in primary afferent nociceptive neurons (Caterina et al., 1997). Synthetic AEA-

This work was supported by MCyT grant BIO 2000-1091-C01 (to E.M.), by European Union grant QLK3-CT-2000-00463 (to E.M. and G.A.), and by INTAS grant 97/1297 (to V.D.M.).

R. S. and M.A.C. contributed equally to this study.

ABBREVIATIONS: AEA, anandamide; 2-AG, 2-arachidonoylglicerol; CB, cannabinoid receptor; FAAH, fatty acid amide hydrolase; VR1, vanilloid receptor type 1; NF-κB, nuclear factor-κB; IκB, κB inhibitor; IKK, IκB kinase; IKC, IκB kinase complex; NIK, NF-κB inducing kinase; TNFα, tumor necrosis factor-α; MEKK, mitogen-activated protein kinase kinase kinase; COX-2, cyclooxygenase 2; HIV-LTR, HIV long terminal repeat; mAb, monoclonal antibody; ATFMK, arachidonoyl trifluoromethyl ketone; *N*-AVAM, *N*-acylvanillamide; DTT, dithiothreitol; NP-40, Nonidet-P40; EMSA, electrophoretic mobility shift assay; RT-PCR, reverse transcriptase-polymerase chain reaction; bp, base pair(s); ERK, extracellular signal-regulated kinase; cyPG, cyclopentenone prostaglandin; SR141716A, *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboximide hydrochloride.

capsaicin "hybrids" have been synthesized, and one of them (arvanil) was found to bind to both VR1 and CB_1 receptors (Melck et al., 1999a).

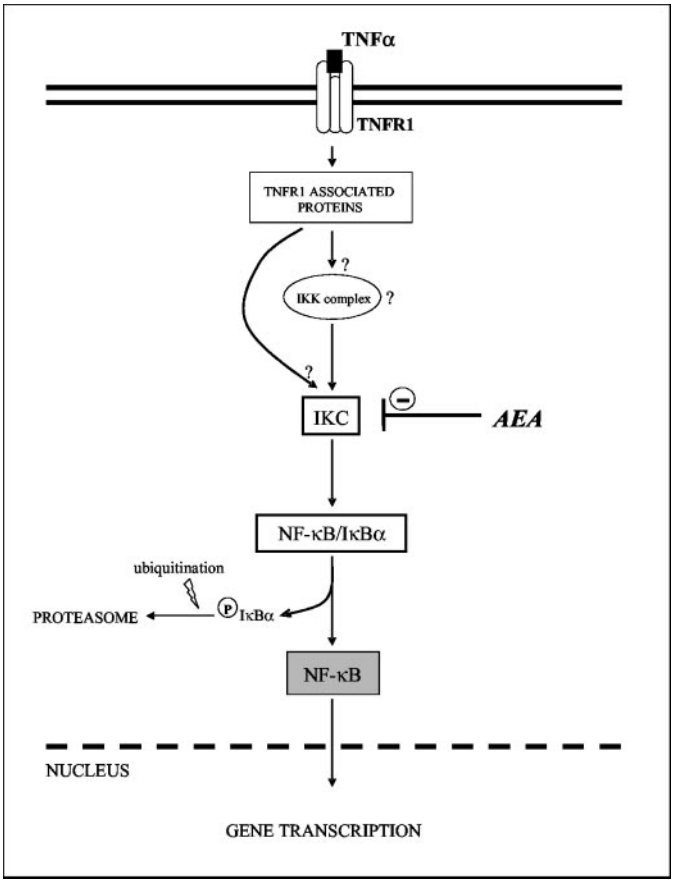
Over the past few years, there has been a growing awareness that AEA and certain synthetic vanilloids exert CB_1- and VR1-independent biological activities (Di Marzo et al., 1999, 2000a). Thus, in $CB1^{+/+}$ mice, the cannabimimetic effects of AEA are not affected by a selective CB_1 receptor antagonist (Adams et al., 1998), whereas in $CB1^{-/-}$ mice AEA stimulates guanosine 5′-*O*-(3-thio)triphosphate binding in brain membranes (Di Marzo et al., 2000b), and arvanil induces inhibition of spasticity and pain via a VR1-independent pathway (Brooks et al., 2002). In addition to the effects on peripheral and central nervous systems, AEA also shows anti-inflammatory, immunomodulatory, and proapoptotic activities (Berdyshev et al., 1997; Maccarrone et al., 2000). Despite the pharmacological relevance of these activities, their mechanistic basis has so far remained elusive.

The transcription factor NF-κB is one of the key regulators of genes involved in the immune/inflammatory response and in survival from apoptosis (Karin and Ben Neriah, 2000). NF-κB is an inducible transcription factor made up of homo- and heterodimers of p50, p65, p52, relB, and c-rel subunits that interact with a family of inhibitory IκB proteins, of which IκBα is the best characterized (Scheme 1). In most cell types, these proteins sequester NF-κB in the cytoplasm by masking its nuclear localization sequence. Stimulation of cells with a variety of physiological or pathogenic stimuli leads to phosphorylation, ubiquitination, and the subsequent degradation of IκBα proteins. The degradation of IκB results in the translocation of NF-κB from the cytoplasm to the nucleus. Phosphorylation of IκBα at serines 32 and 36 is a key step involved in the activation of NF-κB complexes. This event is mediated by IκB kinases (IKKs) (Woronicz et al., 1997), which are formed by a high-molecular-weight complex (IKC) containing at least two kinase subunits (IKKα and IKKβ) and the associated modulatory protein NEMO/IKKγ (Scheme 1). The activation of IKK by different stimuli requires distinct signaling proteins, like the mitogen-activated protein kinase kinase kinase family members NIK, MEKK1, MEKK2, and MEKK3, and also TAK1 and AKT/TPKB kinases (Karin and Ben Neriah, 2000; Hagemann and Blank, 2001). The physiological role of these kinases in signaling through the tumor necrosis factor (TNF) receptor type I has not yet been clarified (Baud and Karin, 2001). Recent data suggest that IKKβ is absolutely required for IκBα phosphorylation and subsequent degradation in TNFα-induced NF-κB activation, whereas IKKα is responsible for the processing of NF-κB2/p100 in a more specialized pathway (Senftleben and Karin, 2002).

NF-κB is highly activated at sites of inflammation in diverse diseases (Tak and Firestein, 2001), where it regulates the transcription of proinflammatory cytokines, chemokines, cytokine receptors, adhesion molecules, and key enzymes in the inflammatory process, such as cycloxygenase-2 (COX-2) and inducible nitric-oxide synthases (Ghosh et al., 1998). Endocannabinoids are also rapidly generated in response to proinflammatory stimulation of immune cells, and they might operate a negative feedback control over the proinflammatory response, possibly by negatively regulating the activation of transcription factors involved in the inflammatory response (Berdyshev et al., 2001b). Furthermore, NF-κB inhibition results in cell apoptosis in some cell systems (Beg et al., 1995) and might be one of the mechanisms underlying AEA apoptotic effects on both immune and nervous cells (Guzman et al., 2001). Previous studies have shown that, in murine macrophages and splenocytes, cannabinoids and the endocannabinoid 2-AG may either activate or inhibit NF-κB activity via cannabinoid receptor- and protein kinase A-dependent mechanisms (Kaminski, 1996; Daaka et al., 1997). Surprisingly, AEA has not yet been investigated as a potential NF-κB modulator. We report here that AEA inhibits the TNFα-induced signals leading to IKK activation, IκBα degradation, and NF-κB activation and that this activity is essentially CB_1- and VR1-independent. The NF-κB inhibitory activity of AEA was retained in certain vanillamides, such as the capsaicin-AEA hybrid arvanil and the fatty acid-based vanilloid olvanil, but not in closely related analogs modified on the acyl chain.

Materials and Methods

Cell Lines and Reagents. The 5.1 clone (obtained from Dr. N. Israël, Institut Pasteur, Paris, France) line is a Jurkat-derived clone stably transfected with a plasmid containing the luciferase gene



Scheme 1. Schematic representation of TNFα signaling pathway from tumor necrosis factor receptor type 1 (TNFR1) to transcription factor NF-κB. TNFα binding to TNFR1 results in the recruitment of TNFR1-associated proteins, which activate the IKC either directly or by means of the activation of a putative IKK kinase(s) (MEKK-1?). Active IKC phosphorylates IκBα, thereby leading to subsequent IκBα ubiquitination and degradation by the proteasome, with subsequent release of NF-κB that can enter the nucleus and start gene transcription. AEA blockade of NF-κB activity could be mediated by inhibition of a cytokine-induced second messenger responsible for activation of IKKβ in IKC.

driven by the HIV-LTR promoter and was maintained in exponential growth in RPMI 1640 medium (BioWhittaker, VerViers, Belgium) supplemented with 10% heat inactivated fetal calf serum, 2 mM L-glutamine, 1 mM HEPES and antibiotics (Invitrogen, Paisley, Scotland), and 200 μg/ml G418. The A549 lung adenocarcinome cell line was obtained from Glaxo SmithKline (London, UK) and was maintained in complete Dulbecco's modified Eagle's media. The anti-IκBα mAb 10B was a gift from R. T. Hay (University of St. Andrews, Fife, Scotland), the mAb anti-tubulin was purchased from Sigma-Aldrich (St. Louis, MO), and the rabbit polyclonal anti-IKK-γ (FL-419) was from Santa Cruz Biotechnology, Inc. (San Diego, CA). The CB1 antagonist SR141716A was purchased from Tocris Cookson (Bristol, UK), and the FAAH inhibitor arachidonoyl trifluoromethyl ketone (ATFMK), anandamide (2-arachidonoyl ethanolamide), and arachidonic acid were from Sigma-Aldrich. Arvanil was purchased from Cayman Chemicals (Ann Arbor, MI). The synthesis of the *N*-AVAMs olvanil, retvanil, retvanil-Ac, farvanil, and ervanil will be published elsewhere (Appendino et al., 2002). *N*-Palmitoyl-, *N*-γ-linolenoyl, and *N*-docosahexaenoyl-ethanolamines were synthesized from the condensation of the corresponding fatty acid chlorides with ethanolamine. [γ-^{32}P]ATP (3000 Ci/mmol) was purchased from ICN Pharmaceuticals (Costa Mesa, CA). The KBF-Luc plasmid, which contains three copies of NF-κB binding site (from major histocompatibility complex promoter), fused to a minimal simian virus 40 promoter driving luciferase. The GST-IκBα$_{(1–54)}$ plasmid, and the expression vectors for IKKα, IKKβ, ΔMEKK1, and NIK have been described elsewhere (Hehner et al., 1999).

Transient Transfections and Luciferase Assays. A549 cells (10^5/ml) were transiently transfected with the KBF-Luc reporter. The transfections were performed using LipofectAMINE PLUS reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations for 24 h. After incubation with AEA for 30 min, transfected cells were stimulated for 6 h with 2 ng/ml TNFα. To determine NF-κB-dependent transcription of the HIV-LTR-Luc 5.1 cells were preincubated for 30 min with AEA and analogs as indicated, followed by stimulation with 2 ng/ml TNFα for 6 h. Then the cells were lysed in 25 mM Tris-phosphate, pH 7.8, 8 mM $MgCl_2$, 1 mM DTT, 1% Triton X-100, and 7% glycerol. Luciferase activity was measured using an Autolumat LB 953 (PerkinElmer Life Science, Boston, MA) following the instructions of the luciferase assay kit (Promega, Madison, WI), and protein concentration was measured by the Bradford method. The background obtained with the lysis buffer was subtracted from each experimental value, the relative luciferase units per microgram of protein were calculated and the specific transactivation expressed as fold induction over untreated cells. All the experiments were repeated at least six times. Statistical analysis was performed using analysis of variance followed by the Student-Newman-Keuls method with values of $p < 0.05$ considered to be significant.

Western Blots. 5.1 cells (1×10^6 cells/ml) were stimulated with TNFα in the presence or absence of AEA for the indicated period of time. Cells were then washed with phosphate-buffered saline and proteins were extracted from cells in 50 μl of lysis buffer (20 mM HEPES, pH 8.0, 10 mM KCl, 0.15 mM EGTA, 0.15 mM EDTA, 0.5 mM Na_3VO_4, 5 mM NaFl, 1 mM DTT, 1 μg/ml leupeptin, 0.5 μg/ml pepstatin, 0.5 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride) containing 0.5% NP-40. Protein concentration was determined by a Bradford assay (Bio-Rad, Hercules, CA), and 30 μg of proteins was boiled in Laemmli buffer and electrophoresed in 10% SDS/polyacrylamide gels. Separated proteins were transferred to nitrocellulose membranes (0.5 A at 100 V; 4°C) for 1 h. The blots were blocked in Tris-buffered saline solution containing 0.1% Tween 20 and 5% nonfat dry milk overnight at 4°C, and immunodetection of specific proteins was carried out with primary antibodies (anti-IκBα, anti-IKKγ, and anti-α-tubulin) using an ECL system (Amersham Biosciences UK, Ltd., Little Chalfont, Buckinghamshire, UK). Densitometry analyses were carried out for the IκBα and α-tubulin blots, and the optical density ratio IκBα/α-tubulin was calculated and assigned the value 1 to untreated cells.

IKK Kinase Assay. Cells were lysed in NP-40 lysis buffer [20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, 0.5 mM sodium vanadate, 10 μg/ml leupeptine, 10 μg/ml aprotinin, 1% (v/v) NP-40, and 10% (v/v) glycerol] for 15 min at 4°C, and after centrifugation for 10 min at 13,000 rpm, the supernatant was incubated with 25 μl of protein A/G-Sepharose and incubated for 2 h on a spinning wheel. After centrifugation, the supernatants were incubated with 2 μg of anti-IKK-γ antibody and 25 μl of protein A/G-Sepharose and incubated for 2 to 4 h on a spinning wheel at 4°C. The precipitate was washed three times in cold lysis buffer and three times in cold kinase buffer (20 mM HEPES/KOH, pH 7.4, 25 mM β-glycerophosphate, 2 mM DTT, and 20 mM $MgCl_2$). The kinase assay was performed in a final volume of 20 μl of kinase buffer containing 40 μM ATP and 5 μCi of γ-32P-ATP and 2 μg of the purified substrate protein GST-IκBα$_{(1–54)}$. After incubation for 20 min at 30°C, the reaction was stopped by the addition of 5× SDS loading buffer. After separation by SDS-polyacrylamide gel electrophoresis the gel was fixed, dried, and exposed to X-ray film.

Isolation of Nuclear Extracts and Mobility Shift Assays. 5.1 or A549 cells (10^6/ml) were stimulated with the agonists in complete medium as indicated. Cells were then washed twice with cold phosphate-buffered saline and proteins from nuclear extracts isolated as described previously (Sancho et al., 2002). Protein concentration was determined by the Bradford method (Bio-Rad). For the electrophoretic mobility shift assay (EMSA), the consensus oligonucleotide probes NF-κB, 5′-AGTTGAGGGGACTTTCCCAGG-3′, and the commercial SP1 site (Promega) were end-labeled with [γ-^{32}P]ATP. The binding reaction mixture contained 3 μg of nuclear extract, 0.5 μg of poly(dI-dC) (Amersham Biosciences Inc., Piscataway, NJ), 20 mM HEPES, pH 7, 70 mM NaCl, 2 mM DTT, 0.01% NP-40, 100 μg/ml bovine serum albumin, 4% Ficoll, and 100,000 cpm of end-labeled DNA fragments in a total volume of 20 μl. When indicated, 0.5 μl of rabbit anti-p50, anti-p65, or preimmune serum was added to the standard reaction before the addition of the radiolabeled probe. For cold competition, a 100-fold excess of the double-stranded oligonucleotide competitor was added to the binding reaction. After 30-min incubation at 4°C, the mixture was electrophoresed through a native 6% polyacrylamide gel containing 89 mM Tris-borate, 89 mM boric acid, and 1 mM EDTA. Gels were pre-electrophoresed for 30 min at 225 V and then for 2 h after loading the samples. These gels were dried and exposed to X-ray film at −80°C.

Reverse Transcriptase-PCR (RT-PCR) Amplification of CB1/CB2 and FAAH mRNA. Total RNA was prepared from 5.1 and A549 cells by the lithium chloride/urea method and digested with DNase. Retrotranscription of mRNA into cDNA was performed in a 20-μl reaction mixture according to the SuperScript II RNase H$^-$ Reverse Transcriptase (Invitrogen) protocol, using 0.5 μg of Oligo(dT)$_{12–18}$ Primer (Invitrogen) for 5 μg of mRNA. The reaction mixture was incubated for 50 min at 42°C, and stopped by heating at 70°C for 15 min, cooled in ice, and stored at −20°C. RT-PCR amplification was performed in a 50-μl PCR reaction mixture containing 0.5 to 2 μl of the retro-transcription mixture, 1× PCR buffer, 1.5 mM $MgCl_2$, 200 μM dNTPs, 10 μM each of 5′ and 3′ primers, and 2.5 units of recombinant *Taq*DNA polymerase (Invitrogen). The mixtures were amplified in a MultiGene cycler IR system (Labnet, Woodbridge, NJ). The primers used were as follows: CB1 sense primer, 5′-CGCAAAGATAGCCGCAACGTGT-3′; CB1 antisense primer, 5′-CAGATTGCAGTTTCTCGCAGTT-3′; CB2 sense primer, 5′-TTTCCCACTGATCCCCAATG-3′; CB2 antisense primer, 5′-AGTTGATGAGGCACAGCATG-3′; FAAH sense primer, 5′-GCCTGGGAAGTGAACAAAGGGACC-3′; FAAH antisense primer, 5′-CCACTACGCTGTCGCACTCCGCCG-3′; β-actin antisense primer, 5′-GCAACTAAGTCATAGTCCGC-3′; and β-actin sense primer, 5′-CTGTCTGGCGGCACCACCAT-3′. The primers chosen for RT-PCR of FAAH and β-actin mRNA amplification included different exons. Therefore, by using these primers any possible DNA contamination

would be detected by the amplification of a higher size band corresponding to an amplicon containing an intron. The amplification profile consisted of an initial denaturation of 2 min at 95°C and then 20 to 35 cycles of 30 s at 95°C, annealing for 30 s at 55°C (CB1 and β-actin) or at 60°C (CB2 and FAAH) and elongation for 1 min at 72°C. A final extension of 10 min was carried out at 72°C. The expected sizes of the amplicons were 244 bp for CB1, 337 bp for CB2, 202 bp for FAAH, and 232 for β-actin. PCR products were electrophoresed on a 1% (w/v) agarose gel and detected by UV visualization.

Results

Transcriptional Activation of NF-κB Is Inhibited by Anandamide. To study the effects of AEA on the activation of the NF-κB pathway, we used the cloned 5.1 cell line that contains the luciferase gene driven by HIV-1-LTR promoter, which is responsive to TNFα through the NF-κB pathway. The HIV-1 promoter contains two NF-κB binding sites that are absolutely required for TNFα-induced transactivation (Alcami et al., 1995). The cells were preincubated with increasing doses of AEA, stimulated with TNFα for 6 h, and finally lysed and the reporter luciferase activity measured. An almost 12-fold increase in luciferase activity over the nonstimulated control cells was noted upon stimulation with TNFα (Fig. 1A). When the cells were pretreated with AEA, TNFα-mediated HIV-1 LTR gene transcription was inhibited in a dose-dependent manner. To further confirm that inhibition of HIV-1-LTR was mediated through the NF-κB sites located in this promoter, the lung carcinoma cell line A549 was transfected with the KBF-Luc plasmid and 24 h later the cells were preincubated with increasing concentrations of AEA for 30 min, and then stimulated with TNFα for 6 h. The dose-dependent inhibition of NF-κB-dependent luciferase expression in A549 cells was very similar to the inhibition observed in the stable cell line (Fig. 1B). To assess the effect of AEA on NF-κB DNA binding activity, an EMSA was performed on nuclear extracts of 5.1 cells preincubated with increasing doses of AEA and stimulated for 30 min with 2 ng/ml TNFα. As shown in Fig. 2A, 5.1 cells pretreated with AEA exhibited a dose-related decrease of TNFα-induced NF-κB binding activity. The DNA binding specificity was studied by supershift experiments with specific anti-p50 and anti-p65 (RelA) antibodies and by cold competition experiments with unlabeled competitors and identified the heterodimer p50/p65 as the main complex activated by TNFα in 5.1 cells (Fig. 2B). As expected, AEA also inhibited the NF-κB binding activity in A549 cells, and Fig. 2C shows that this endocannabinoid at 25 μM greatly reduced the binding to DNA of this transcription factor in a kinetic study. Conversely, no significant effect by AEA was observed on binding activity at the Sp1 site (data not shown).

AEA Inhibits IκBα Degradation and IKK Activation. In light of the previous results we performed experiments to identify the molecular target for the AEA effects on the NF-κB activation pathway. 5.1 cells were stimulated with TNFα for 15 min in the absence or presence of increasing concentrations of AEA, and total cell extracts were analyzed in parallel for IκBα degradation and IKK activation. The TNFα-induced degradation of IκBα was completely inhibited in a dose-dependent manner by AEA, which did not affect the steady-state levels of α-tubulin (Fig. 3A). Because the degradation of IκB proteins was shown to occur after signal-induced phosphorylation of IκB proteins at specific serine residues, catalyzed by IKKs present in the IKC (Karin and Ben Neriah, 2000), we tested whether the prevention of IκBα degradation was due to an impaired kinase activity of the IKC. Endogenous IKC was isolated by immunoprecipitation with an anti-IKKγ antibody, and its activity was analyzed by immune complex kinase assays using recombinant IκBα protein as substrate. TNFα stimulation in 5.1 cells induced an approximately 10-fold increase in IKK activity compared with unstimulated cells, and this activity was dose dependently inhibited in the presence of increasing doses of AEA (Fig. 3B).

Anandamide Prevents MEKK-1, NIK, and IKKα-Induced NF-κB Activation. Because AEA impaired TNFα-induced activation of IKK activity, obvious direct candidates for the inhibitory activity of AEA are the IKKs. This possibility was tested in A549 cells transiently transfected with the KBF-Luc plasmid alone or in combination with expression vectors encoding IKKα and IKKβ. The IKKβ-induced NF-κB activation was efficiently inhibited by AEA in a concentration-dependent manner (Fig. 4B), very similar to the one observed for AEA inhibition of TNFα-induced NF-κB activation (Fig. 1B). In addition, NF-κB-dependent transcription induced by overexpression of IKKα was also significantly

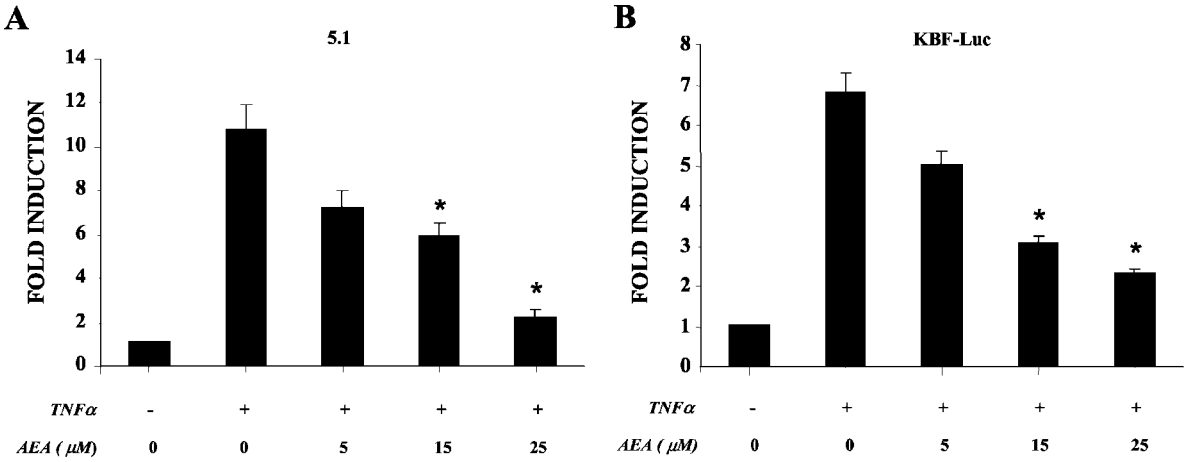


Fig. 1. Anandamide inhibits NF-κB-dependent luciferase gene expression. 5.1 cells (A) or KBF-Luc transiently transfected A549 cells (B) were pretreated with different doses of anandamide and treated with TNFα for 6 h, after which luciferase activity was measured. The results show fold induction ± S.D. over the control ($n = 6$; *, $p < 0.05$). The relative light units in control cells were 256 ± 43/μg of protein.

inhibited by AEA, although to a lesser extent (Fig. 4A). We next wanted to assess whether AEA could affect the upstream regulatory IKK kinases, NIK, and MEKK1. To address this question, A549 cells were transiently transfected with expression vectors encoding NIK and the catalytic domain of MEKK1 (MEKK1Δ), and KBF-Luc as reporter. As shown in Fig. 4C, NF-κB-dependent transcription induced by MEKK1 was partially suppressed by the presence of AEA; conversely, increasing doses of AEA completely inhibited the NF-κB activity induced by NIK. Together, these results strongly suggest that the main target for AEA in the NF-κB signaling pathway lies at the level of IKKβ-induced stimulation of IKC (Scheme 1).

Cannabinoid Receptors and FAAH Do Not Mediate AEA-Induced NF-κB Inhibition. To assess a possible involvement of cannabinoid receptors, or of the hydrolysis of AEA, in the inhibitory effect on NF-κB activity, we carried out a series of experiments. Because AEA could inhibit TNFα-mediated NF-κB activation in both A549 and 5.1 cells, the expression of both cannabinoid receptors and FAAH in these cell lines was investigated by RT-PCR. The results showed that 5.1 only expressed mRNA for CB_2, and A549 cells only mRNA for CB_1. Both cell lines expressed a similar amount of FAAH mRNA (Fig. 5). Thus, 5.1 cells were preincubated with increasing concentrations of AEA and the FAAH-specific inhibitor ATFMK at a concentration that has been previously shown to be effective to prevent AEA enzymatic hydrolysis (Deutsch et al., 1997) and then stimulated with TNFα for 6 h. AEA-mediated inhibition of TNFα signaling was not influenced by the presence of ATFMK, suggesting that hydrolysis of AEA is not required for its NF-κB inhibitory activity (Fig. 6A). Similar results were obtained in the A549 cell line transfected with the KBF-Luc plasmid (Fig. 6B). In addition, the pretreatment of 5.1 cells with increasing concentrations of arachidonic acid did not inhibit the transactivation of the HIV-LTR promoter induced by TNFα, thus ruling out the possibility that the NF-κB inhibitory effect of AEA is mediated by one of the two products of its hydrolysis by FAAH (Fig. 6A). Although the lack of expression of CB_1 in 5.1 cells rules out the involvement of CB_1 in AEA-mediated NF-κB inhibition, both the 5.1 cell line and the A549 cells transiently transfected with KBF-Luc were stimulated with TNFα in the presence or absence of the CB_1 antagonist SR141716A (2.5 μM, A549 cells only express CB1, as shown in Fig. 5). The results showed that AEA-inhibition of NF-κB activation was not affected by the presence of this CB_1 antagonist neither in A549 transfected cells nor in the CB_1 negative 5.1 cell line (Fig. 6, C and D).

Chemical Modification of AEA Influences Its Capability to Inhibit NF-κB Activation. A series of AEA analogs were also tested on TNFα-stimulated NF-κB activity (Fig. 7A). Saturation plus shortening of the fatty acyl chain led to the anti-inflammatory compound *N*-palmitoylethanolamine (Lambert and Di Marzo, 1999), which did not inhibit TNFα-induced NF-κB activation. Also, introduction of ω-3 polyunsaturated fatty acids resulted in no NF-κB inhibitory activity. However, substitution of the ethanolamine polar group with a vanillylamine group, as in arvanil (Di Marzo et

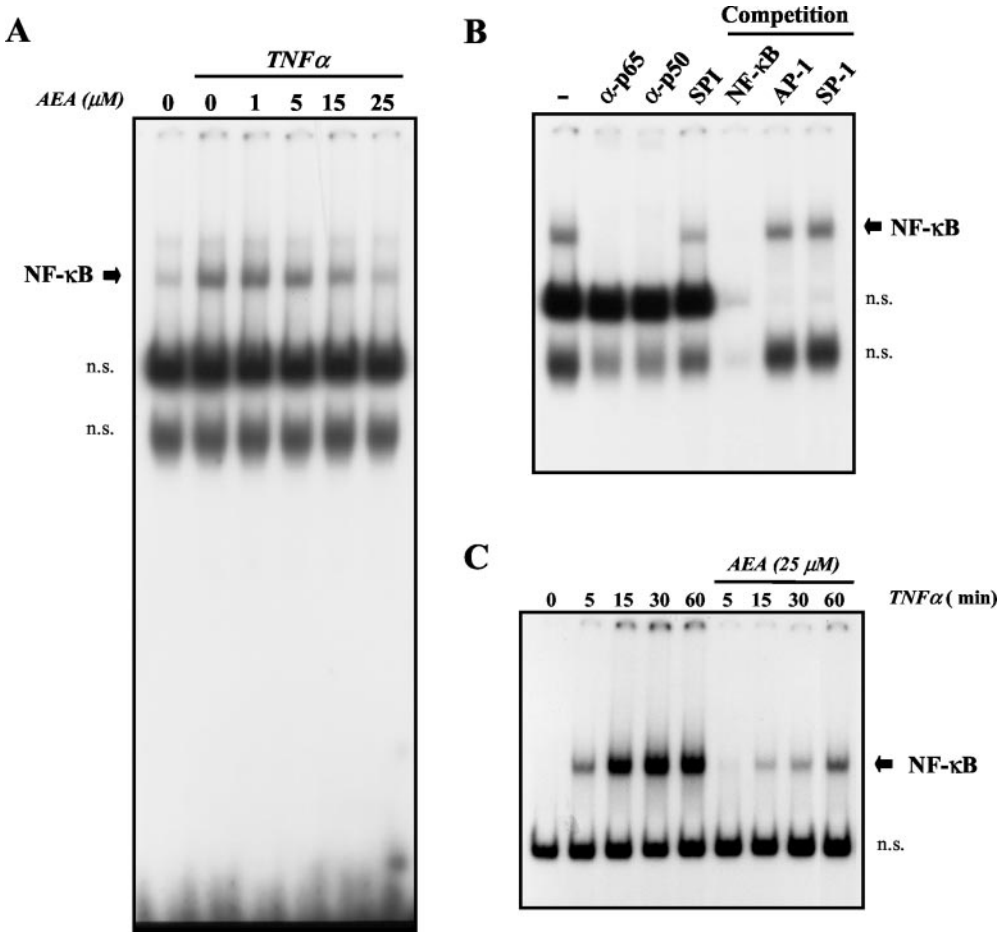


Fig. 2. Effects of AEA in NF-κB/DNA binding. 5.1 cells, either untreated or pretreated with AEA at the doses indicated, were incubated with TNFα for 30 min. The nuclear extracts from these cells were then assayed by EMSA using γ-^{32}P-labeled NF-κB oligonucleotide (A). Cold competition and supershift experiments were assayed using nuclear extracts from TNFα treated cells (B). A549 cells, treated or untreated with AEA at the indicated dose, were incubated in the presence of TNFα for different periods of time. Nuclear extracts were then assayed by EMSA for NF-κB binding activity (C).

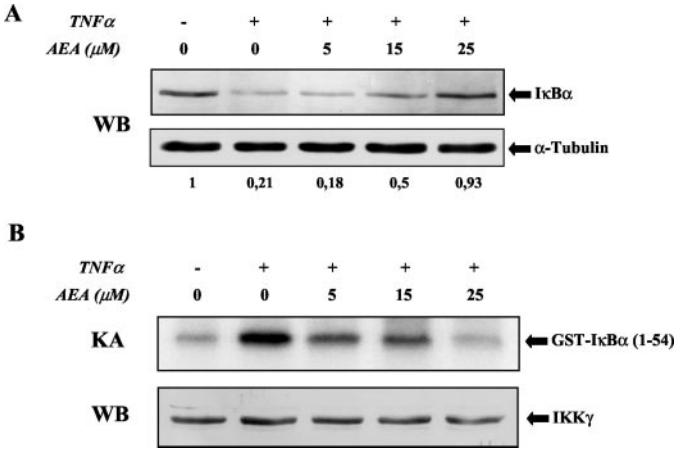


Fig. 3. Inhibition of IκBα degradation and IKK activity by AEA. 5.1 cells were stimulated as indicated and lysed in NP-40 lysis buffer. One aliquot of the extracts was used for Western blot analysis for IκBα and α-tubulin expression (A). The rest of the lysate was immunoprecipitated with α-IKKγ and assayed for IKC kinase activity using GST-IκBα$_{(1-54)}$ as substrate (B, top). The steady levels of IKKγ protein were determined by Western blot using a separate aliquot of proteins (30 μg) from the immunoprecipitated lysate (B, bottom).

al., 2000a), slightly enhanced the activity of AEA. Partial saturation of arvanil, as in ervanil (the vanillamide of erucic acid), again resulted in an inactive compound. However, the corresponding oleyl analog, olvanil, was as potent as arvanil despite the presence of a shorter and less saturated fatty acyl chain. Introduction of an allylic hydroxyl the distal homoallylic position (C-12) of olvanil (rinvanil; vanillamide of ricinoleic acid) almost completely abrogated the inhibitory effect of this compound at the doses tested, whereas the acetylation of rinvanil restored in part the inhibitory activity on NF-κB activity. Finally, branching/shortening of the aliphatic chain of olvanil, as in farvanil (vanillamide of farnesic acid) and retvanil (vanillamide of retinoic acid), abolished the inhibitory NF-κB activity in TNFα-stimulated 5.1 cells (Fig. 7B). These data support the involvement of a non-CB$_1$, non-VR1 structure-sensitive binding site in the NF-κB activity of AEA and arvanil. This view is in accordance with the observation that, despite a slight activity on CB$_1$ (*N*-γ-linolenoyl and *N*-docosahexaenoyl-ethanolamine; V. Di Marzo, unpublished data) or a high potency on VR1 (rinvanil, retvanil, farvanil; Appendino et al., 2002), many analogs of AEA investigated here were totally inactive on NF-κB. Remarkably,

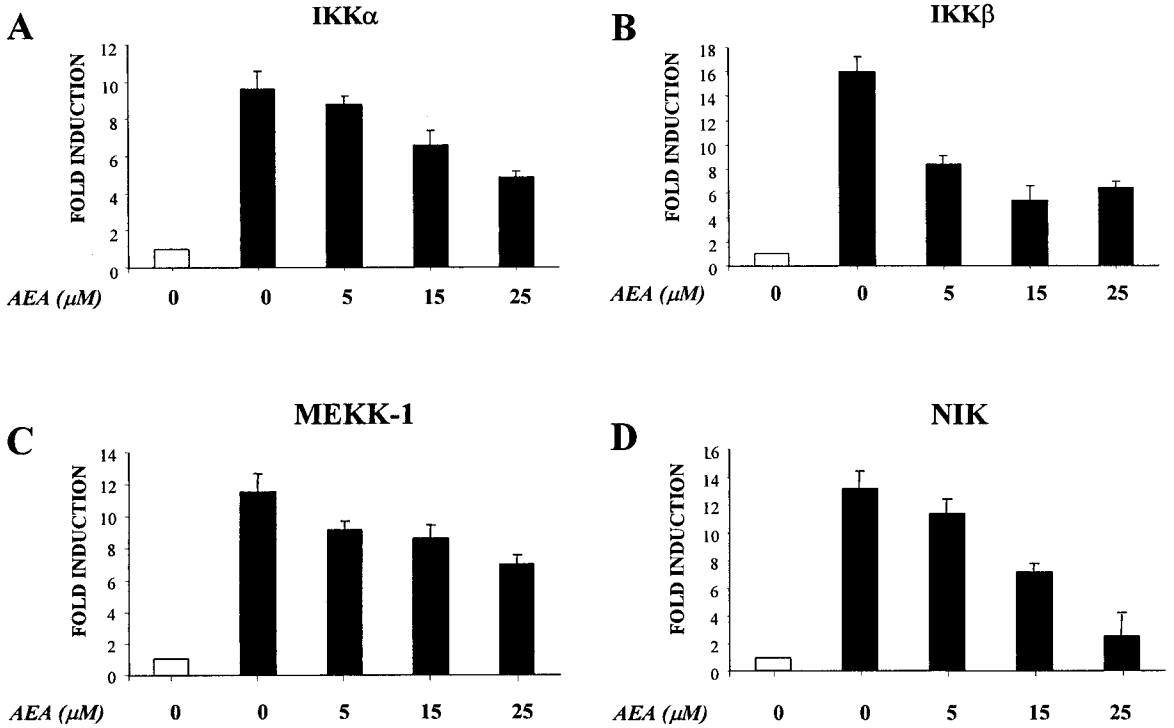


Fig. 4. Effect of AEA in IKKα, IKKβ, NIK, and MEKK NF-κB-dependent transcription. A549 cells were transiently transfected with KBF-Luc alone or in combination with either IKKα (A), IKKβ (B), ΔMEKK-1 (C), or NIK (D) expression vectors. After 24 h of transfection cells were stimulated with different doses of AEA for 6 h and luciferase activity was assayed. The results show fold induction ± S.D. over unstimulated cells. The data are representative of six different experiments. White bars represent transfected cells with empty vectors and the KBF-Luc plasmid.

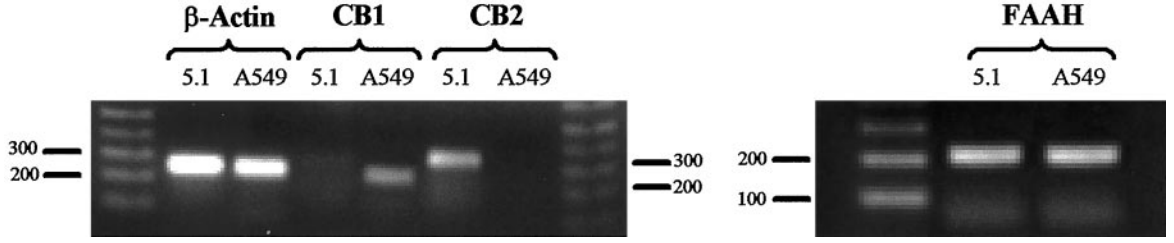


Fig. 5. FAAH, CB$_1$, and CB$_2$ mRNA expression pattern. RT-PCR results for FAAH and CB$_{1/2}$ receptors in 5.1 and A549 cell lines.

although arvanil was shown to activate both CB_1 and VR1 receptors (Melck et al., 1999a; Ross et al., 2001), some of its biological functions were suggested to be independent of these receptors (Brooks et al., 2002).

Discussion

Anandamide, the amide of arachidonic acid with ethanolamine, was the first ligand of the cannabinoid receptors to be described, and exhibits anti-inflammatory and proapoptotic activities (De Petrocellis et al., 2000; Guzman et al., 2001). It has been suggested that, in response to proinflammatory cell stimulation, endogenous cannabinoids may be rapidly generated and secreted, thereby resulting in the stimulation of cannabinoid receptors in adjacent cells and subsequent down-regulation of the inflammatory response (Berdyshev et al., 2001b). Thus, it was shown that lipopolysaccharide increases AEA levels by down-regulating FAAH expression in human peripheral lymphocytes, whose apoptosis is induced by AEA via a non-CB_1-mediated mechanism (Maccarrone et al., 2001). AEA produced by lymphoid cells may participate also in the lipopolysaccharide-induced septic shock by inducing vasodilatation through a direct action on vascular smooth muscle (Varga et al., 1998). However, it is also possible that new synthesized AEA may limit the proinflammatory response by direct inhibition of proinflammatory cytokine release (Berdyshev et al., 1997, 2001), or by down-regulation of inducible nitric-oxide synthase in the cardiovascular endothelium (Stefano et al., 1998). Anandamide also activates vanilloid VR1 receptors (Zygmunt et al., 1999), thereby inducing apoptosis of several cell types (Maccarrone et al., 2000). However, there is growing evidence that some of the pleiotropic effects of anandamide are independent of both cannabinoid and vanilloid receptors.

Activation of the NF-κB/Rel transcription family plays a central role in inflammation and apoptosis through its ability to induce transcription of genes for proinflammatory and apoptosis-survival proteins (Tak and Firestein, 2001). The pleiotropic and not yet fully rationalized pattern of activities of AEA provided a rationale to investigate the effect of AEA in the NF-κB activation pathway. Our results clearly demonstrate that AEA is a selective inhibitor of TNFα-mediated NF-κB activation through a noncannabinoid receptor- and non-VR1-mediated mechanism. Because IKKα and β are the two kinases of the IKC that phosphorylate the NF-κB inhibitory protein IκBα, and we found a significantly higher inhibitory effect of AEA on IKKβ-induced NF-κB activation, it is likely that the main molecular target for the inhibitory activity of AEA in TNFα-signaling is the IKKβ subunit, a specific point that requires further analyses (see proposed model in Scheme 1). Although the physiological signaling activated by TNFα in the NF-κB pathway probably does not involve NIK (Baud and Karin, 2001), it is clear that overexpression of this kinase also targets both catalytic subunits of the IKC, thus explaining the inhibitory effect of AEA on NIK-induced NF-κB activation. Moreover, the inhibitory effects of AEA seem to be specific for the NF-κB signaling pathway because we did not find any inhibition of other relevant mitogen-activated protein kinases (ERK and p38) in TNFα-stimulated Jurkat cells (data not shown). Indeed, AEA was shown to slightly activate ERK and induce AP-1 activation through a CB1-independent pathway in the mouse JB6 cell line (Berdyshev et al., 2001a), and to activate ERK and p38 kinases in a CB1-dependent manner in MCF-7 cells and neurons (Melck et al., 1999b; Derkinderen et al., 2001).

The molecular mechanism by which AEA inhibits TNFα-mediated IKK activation, however, remains to be elucidated. One possibility is that intracellular AEA serves as a substrate for the enzyme COX-2 (Yu et al., 1997), leading to the generation of different prostaglandins that may in turn inhibit NF-κB. Indeed, it has been shown that cyclopentenone prostaglandins (cyPGs) can inhibit NF-κB activation in TNFα-stimulated cells (Rossi et al., 1997). However, the NF-κB inhibitory activity of prostaglandins is restricted to a

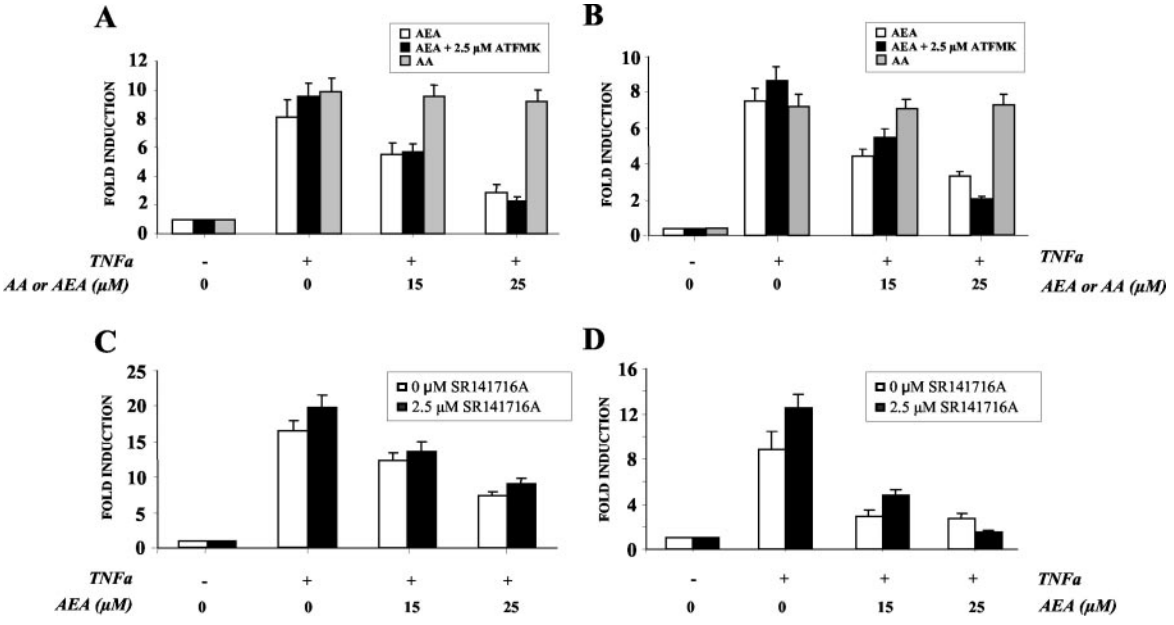


Fig. 6. Cannabinoid receptors or FAAH do not mediate AEA-induced NF-κB inhibition. Effect of ATFMK (FAAH inhibitor) in NF-κB inhibition by AEA in 5.1 cells (A) and in KBF-Luc transfected A549 cells (B). Effect of the CB_1 antagonist (SR141716A) on NF-κB inhibition by AEA in 5.1 cells (C) and in KBF-Luc transfected A549 cells (D). ATFMK and SR141716A were added to the cell cultures 15 min before AEA pretreatment.

subset of cyPGs (Rossi et al., 2000), which has been shown to be produced at a delayed point in the inflammatory process (Gilroy et al., 1999). Yet, the finding that AEA inhibits IKC activation within 15 min of stimulation, whereas arachidonic acid does not inhibit the TNFα-mediated NF-κB activation, strongly suggests that the NF-κB inhibitory activity of AEA is not mediated by the generation of cyPGs. Furthermore, the inhibitory effect of AEA on NF-κB could not be related to the generation of its hydrolysis products, because it was not affected by inhibitors of FAAH, the enzyme responsible for the AEA metabolism. In full agreement with this view, inhibition of TNF-mediated NF-κB activation in 5.1 cells was also shown by the more metabolically stable AEA analog arvanil, as well as by olvanil, a compound lacking the arachidonoyl motif.

The narrow structure-activity relationships for the inhibition of NF-κB by AEA suggest the interaction of this compound with a specific site, and not simply a nonspecific "membrane perturbation" effect. This finding has great relevance for the endocannabinoid field, where the discovery of new molecular targets for AEA has been long pursued. Thus, AEA inhibits T-type calcium channels (Chemin et al., 2001) and is also a selective blocker of the K^+ channel TASK-1 (Maingret et al., 2001), but the possibility that AEA inhibits NF-κB by a selective block of certain ion channels requires further investigation. Another attractive possibility is that AEA inhibition of NF-κB is mediated by the same noncannabinoid, nonvanilloid receptors that have been proposed to be stimulated by AEA, arvanil, and other long-chain *N*-AVAMs (Di Marzo et al., 2001, 2002). This possibility is supported by our finding that also arvanil and olvanil inhibit TNF-α-mediated NF-κB activation, as well as by recent data showing that other lipids containing a vanillyl group, including capsaicin itself and capsiate, inhibit NF-κB via a non-VR1-mediated mechanism (Oh et al., 2001; Sancho et al., 2002). Finally, in further support of a mechanism mediated by a cell type-specific site of action, we found that AEA could not inhibit TNFα or phorbol 12-myristate 13-acetate-induced activation of NF-κB in HeLa cells (data not shown).

The relatively high concentrations of AEA required for NF-κB inhibition should not be seen as evidence against a receptor-mediated mechanism of action. In fact, the other endocannabinoid 2-AG, as well as more metabolically stable cannabinoid receptor agonists, were shown to exert a similar

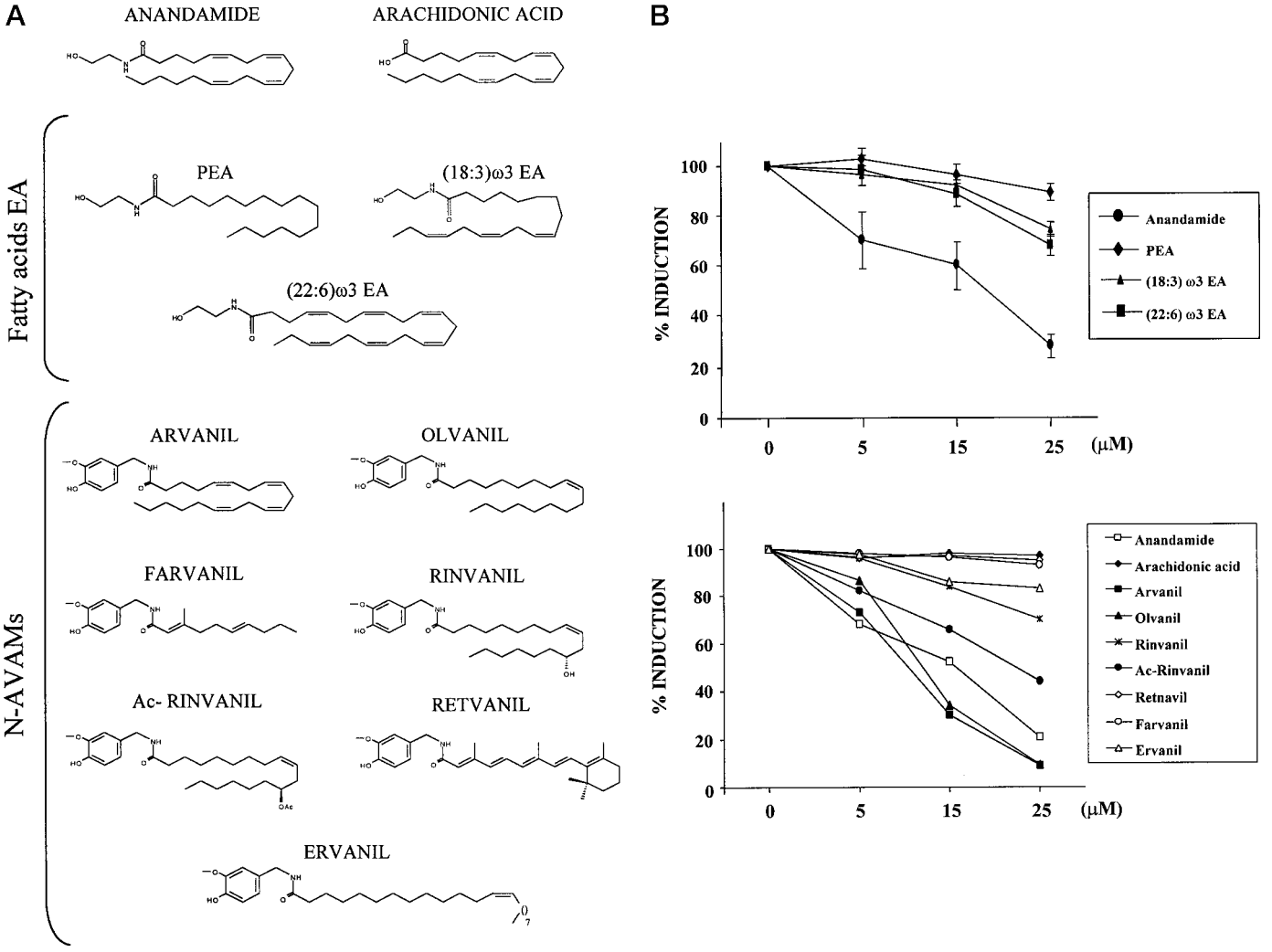


Fig. 7. Effect of *N*-acyl-vanillamines and ethanolamines in TNFα-mediated NF-κB activation. Chemical structures of different ethanolamines and *N*-AVAMs (A). Percentage of NF-κB-dependent luciferase activity induction in 5.1 cells pretreated either with different ethanolamines (top) or *N*-AVAMs (bottom) at the indicated doses and treated with TNFα for 6 h (B).

effect at identical concentrations, and through the interaction with cannabinoid receptors (Condie et al., 1996; Ouyang et al., 1998; Herring and Kaminski, 1999). The possibility that experimental and methodological factors prevent the observation of the effect of cannabimimetic lipophilic substances on NF-κB at submicromolar concentrations should be taken into account. Furthermore, the effect of AEA on VR1 was also shown to occur at >1 μM concentrations, but further experiments have shown that the threshold for VR1 activation by AEA can be sensibly lowered by several regulatory factors (Di Marzo et al., 2001). For example, the action of AEA at both VR1 and T-type channels is exerted at a site on the cytosolic side of these membrane proteins and is controlled by AEA-facilitated transport into the cell (Chemin et al., 2001; De Petrocellis et al., 2001). It is therefore possible that the AEA membrane transporter is lacking, or at least not very active, in the cells used in our assays, and that higher extracellular concentrations of AEA are required for the observation of its intracellular effects. Furthermore, the relatively low potency of AEA against NF-κB activity should not diminish the potential physiological and pathological importance of our observations. In fact, although nanomolar extracellular concentrations of AEA are expected to occur under physiological conditions, the intracellular concentrations might be much higher, particularly under pathological conditions leading to inflammation and apoptosis, such as cell injury and tissue damage (Berdyshev et al., 2000, 2001a).

Taken together, our observations show that AEA exhibits NF-κB inhibitory activity and that this effect is not mediated by the interaction of AEA with cannabinoid or vanilloid receptors. This activity might underlie some of the known anti-inflammatory and proapoptotic effects of AEA and provides a rationale for the synthesis of AEA analogs endowed with selective anti-inflammatory and anticancer properties.

Acknowledgments

We thank Dr. Nicole Israël (Institute Pasteur, Paris, France) for the 5.1 cells; Dr. Alain Israël (Institute Pasteur) for the anti-p50 and anti-p65 antisera; Dr. R. T. Hay (CBMS, University of St. Andrews, Fife, Scotland) for the mAb 10B; and colleagues Drs. D. Goeddel (San Francisco, CA), D. Wallach (Rehovot, Israel), and L. Schmitz (Bern, Switzerland) for providing plasmids. We are grateful to Dr. Filomena Fezza (Instituto di Chimica Biomolecolare) and to Dr. Nives Daddario (Dipartimento di Scienze Chimiche Alimentari Farmaceutiche e Farmacologiche) for the synthesis of *N*-acylethanolamines and *N*-acylvanillamines, respectively. Finally, we thank Carmen Cabrero-Doncel for assistance with the manuscript.

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