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Anandamide Regulates Keratinocyte Differentiation by Inducing DNA Methylation in a CB1 Receptor-dependent Manner*

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Anandamide (arachidonoylethanolamide, AEA) belongs to an important class of endogenous lipids including amides and esters of long chain polyunsaturated fatty acids, collectively termed "endocannabinoids." Recently we have shown that AEA inhibits differentiation of human keratinocytes, by binding to type-1 cannabinoid receptors (CB1R). To further characterize the molecular mechanisms responsible for this effect, we investigated the expression of epidermal differentiation-related genes after AEA treatment. We observed that keratin 1 and 10, transglutaminase 5 and involucrin are transcriptionally down-regulated by AEA. Most importantly, we found that AEA is able to decrease differentiating gene expression by increasing DNA methylation in human keratinocytes, through a p38, and to a lesser extent p42/44, mitogen-activated protein kinase-dependent pathway triggered by CB1R. An effect of AEA on DNA methylation because of CB1R-mediated increase of methyltransferase activity is described here for the first time, and we believe that the importance of this effect clearly extends beyond the regulation of skin differentiation. In fact, the modulation of DNA methylation by endocannabinoids may affect the expression of a number of genes that regulate many cell functions in response to these substances.

Anandamide (arachidonoylethanolamide, AEA)[4] belongs to an important class of endogenous lipids including amides and esters of long chain polyunsaturated fatty acids, collectively termed "endocannabinoids" (1, 2). AEA is released from depolarized neurons, endothelial cells, and macrophages (3), and mimics the pharmacological effects of Δ9-tetrahydro-cannabinol, the active principle of hashish and marijuana (4). Extracellular AEA binds to type-1 and type-2 cannabinoid receptors (CB1R and CB2R) (4), thus playing many actions in the central nervous system and in the periphery (2, 3). The endogenous concentration of AEA is controlled *in vivo* through degradation by fatty acid amide hydrolase (FAAH) (5), preceded or not by cellular uptake through a putative AEA membrane transporter (6, 7). The main checkpoint in AEA synthesis seems to be the *N*-acyl-phosphatidylethanolamines (NAPE)-hydrolyzing phospholipase D (NAPE-PLD), which releases on demand AEA from membrane NAPEs (8). However, additional metabolic routes seem to contribute to the synthesis of AEA (9, 10). Another major endocannabinoid is 2-arachidonoylglycerol (2-AG), for which specific metabolic enzymes have been recently discovered (11, 12); the physiological relevance of these enzymes is the subject of intense investigation (13). Together with AEA, 2-AG and congeners, the proteins that bind and metabolize these substances form the endocannabinoid system (ES) (3, 14). Full and functional ES has been found virtually in all tissues and its relevance within the central nervous system has been clearly demonstrated (15). Peripheral endocannabinoids seem to play a crucial role in modulating the autonomic nervous, reproductive, endocrine, and immune systems (16–19), as well as in controlling pain initiation (20, 21). Recently, attention has been focused on the possible role of AEA and other endocannabinoids in regulating cell growth and differentiation, and collected evidence suggests that AEA might have pro-apoptotic activity (22, 23). In this context, we have shown that human keratinocytes have a functional ES that enables them to bind and metabolize AEA; moreover, ES was shown to be implicated in the control of epidermal differentiation, through a CB1R-dependent mechanism (24). The epidermis, which forms the uppermost compartment of the skin, represents a barrier against the environment, provided by terminally differentiating keratinocytes (25, 26). Epidermal differentiation begins with the migration of keratinocytes from basal layer, composed of proliferating cells, and ends with the formation of the cornified cell envelope, an insoluble protein structure found in differentiated keratinocytes (27). Cell proliferation and differentiation occur sequentially and are charac-

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[4] The abbreviations used are: AEA, arachidonoylethanolamide; CB1/2R, type-1/2 cannabinoid receptor; FAAH, fatty acid amide hydrolase; NAPE, *N*-acyl-phosphatidylethanolamines; NAPE-PLD, NAPE-hydrolyzing phospholipase D; 2-AG, 2-arachidonoylglycerol; ES, endocannabinoid system; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; 5AC, 5-azacytidine; NADA, *N*-arachidonoyldopamine; K1/10, keratin 1/10; TGase 5, transglutaminase 5; MSP, methylation-specific PCR; DNMT, DNA methyltransferase; EDC, epidermal differentiation complex; ACEA, arachidonoyl-2-chloroethylamide; MAPK, mitogen-activated protein kinase; RT, reverse transcription.

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terized by the expression of specific proteins, such as keratins and transglutaminases (28, 29). Activation of several keratinocyte differentiation genes requires the opening of chromatin structure and demethylation of specific genomic promoter regions. Variation in overall DNA methylation between differentiated and undifferentiated cells has been reported in a number of different models (30, 31), and DNA of differentiated keratinocytes has been shown to contain less 5-methylcytosine than DNA of undifferentiated keratinocytes (32). Moreover, agents known to inhibit DNA methylation (*i.e.* 5-azacytidine, 5AC) and histone deacetylation (*i.e.* sodium butyrate, NaB) are also known to inhibit growth and to promote differentiation of keratinocytes (33–35). We have previously reported that differentiating keratinocytes have decreased levels of endogenous AEA, because of increased degradation of this lipid through FAAH. In addition, we have shown that exogenous AEA inhibits keratinocyte differentiation *in vitro*, leading to a CB1R-dependent reduction of cornified envelope formation and transglutaminase activity (24). On the other hand, it has been shown that endocannabinoids regulate neuritogenesis, axonal growth, and synaptogenesis in differentiated neurons (36, 37), leading to the hypothesis that endocannabinoids are general signaling cues responsible for the regulation of cellular proliferation and differentiation. To evaluate the molecular mechanisms underlying the influence of endocannabinoids, and in particular of AEA, on cell differentiation, we sought to investigate the effects of exogenous AEA on the gene expression pattern of differentiating human keratinocytes.

EXPERIMENTAL PROCEDURES

Materials—Chemicals were of the purest analytical grade. AEA, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), 5-azacytidine (5AC), and *N*-arachidonoyldopamine (NADA) were purchased from Sigma. *S*-Adenosyl-L-[*methyl*-^{3}H]methionine was from Amersham Biosciences (Buckinghamshire, UK), 2-arachidonoylglycerol (2-AG) was from Research Biochemicals International (Natick, MA). Arachidonoyl-2-chloroethylamide (ACEA) was purchased from Cayman Chemical (Ann Arbor, MI). PD98059 and SB203580 were from Calbiochem (San Diego, CA). *N*-Piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole-carboxamide (SR141716) was a kind gift from Sanofi-Aventis (Montpellier, France).

Cell Culture and Treatments—HaCaT cells were grown in a 1:1 mixture of minimum essential medium and Ham's F-12 medium (Invitrogen, Berlin, Germany), supplemented with 10% fetal calf serum and 1% nonessential amino acids, at 37 °C in a 5% CO_2 humidified atmosphere. Cell differentiation was induced by treating HaCaT cells with TPA (10 ng/ml) plus $CaCl_2$ (1.2 mM) for 5 days (38). AEA and related compounds were added at the indicated concentrations directly to the serum-free culture medium, at the same time as TPA plus calcium (24). Culture medium containing freshly prepared AEA and the other reagents was changed daily during the treatment. Culture medium containing vehicles alone was added to controls under the same conditions (24). After each treatment, cell viability was determined by Trypan Blue dye exclusion. The treatment of differentiating HaCaT cells with 5AC was performed by seeding 3×10^6 cells in 100-cm^2 tissue culture flasks. After 24 h, cells were exposed to 1 μM 5AC for 5 days.

Real-time PCR Assay—RNA was extracted using the RNeasy extraction kit (Qiagen, Crawley, UK) from proliferating and differentiating HaCaT cells, following the manufacturer's instructions. RT-PCR reactions were performed using the RT-PCR SuperScript III Platinum Two-Step qRT-PCR Kit (Invitrogen, Carlsbad, CA). 1 μg of total RNA was used to produce cDNA with 10 units/μl SuperScript III reverse transcriptase, in the presence of 2 units/μl RNaseOUT, 1.25 μM oligo(dT)$_{20}$, 1.25 ng/μl random hexamers, 5 mM $MgCl_2$, 0.5 mM dNTP mix, and DEPC-treated water. The reaction was performed using the following RT-PCR program: 25 °C for 10 min, 42 °C for 50 min, 85 °C for 5 min, then, after addition of 0.1 units/μl of *Escherichia coli* RNase H, the product was incubated at 37 °C for 20 min. For expression studies, the target transcripts were amplified in ABI PRISM 7700 sequence detector system (Applied Biosystems, Foster City, CA), using the following primers: keratin 10 (K10) F1 (5′-ACGAGGAGGAAATGAAAGAC-3′), K10 R1 (5′-GGACTGTAGTTCTATCTCCAG-3′); keratin 1 (K1) F1 (5′-AGAAAGCAGGATGTCTGG-3′), K1 R1 (5′-AAACAAACTTCACGCTGG-3′); involucrin (INV) F1 (5′-CTCTGCCTCAGCCTTACT-3′), INV R1 (5′-GCTGCTGATCCCTTTGTG-3′); transglutaminase 5 (TG5) F1 (5′-TCAGCACAAAGAGCATCCAG-3′), TG5 R1 (5′-TTCAGGGAGACTTGCACCAC-3′); β-actin F1 (5′-TGACCCAGATCATGTTTGAG-3′) and β-actin R1 (5′-TTAATGTCACGCACGATTTCC-3′). Actin was used as housekeeping gene for quantity normalization. One microliter of the first strand cDNA product was used for amplification in triplicate in a 25-μl reaction solution containing 12.5 μl of Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) and 10 pmol of each primer. The following PCR program was used: 95 °C for 10 min; 40 amplification cycles at 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s.

Immunoblotting Analysis—HaCaT cell protein extracts (20 μg per lane) were loaded onto 10% SDS-polyacrylamide gels and blotted onto polyvinylidene difluoride sheets (Amersham Biosciences). Filters were blocked with 10% nonfat dried milk and 5% bovine serum albumin for 2 h, and then were incubated for 2 h with rabbit anti-K10 (diluted 1:1000 in blocking solution; Berkeley Antibody Company, Richmond, CA) and mouse anti-actin (1:1000 in blocking solution; Santa Cruz Biotechnology, Santa Cruz, CA) antibodies. After three washes with phosphate-buffered saline + 0.05% Tween 20, filters were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (1:2000 in blocking solution; Santa Cruz Biotechnology) for 1 h. Detection was performed using West Dura Chemiluminescence System (Pierce, Rockford, IL).

DNase I Sensitivity Assay—The procedure for the isolation of nuclei was reported previously (39). A total of 5×10^5 nuclei in DNase I buffer (10 mM Tris-HCl, 10 mM NaCl, 3 mM $MgCl_2$, 100 mM $CaCl_2$, pH 7.4) were treated with increasing amounts (0, 0.5, 1, 2, and 10 units) of DNase I (Roche Applied Science) in a reaction volume of 200 μl for 30 min at 25 °C. The reactions were terminated by adding an equal volume of stop solution (1% sodium dodecyl sulfate, 0.1 M NaCl, 50 mM Tris-HCl, pH 8.0, and 10 mM EDTA), containing 1 mg of proteinase K per ml,

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followed by incubation at 55 °C for 2 h. DNA was extracted with phenol-chloroform and was ethanol-precipitated. The K10 gene was amplified by PCR (50 ng/reaction; 30 cycles) with the primers K10P WF and K10P WR, described below for the methylation-specific PCR. The PCR products were separated on 1.6% agarose gel and stained with ethidium bromide.

Bisulfite DNA Modification—Genomic DNA was isolated from HaCaT cells using DNeasy kit (Qiagen, Crawley, UK). Sodium bisulfite treatment of DNA was performed using the CpGenome DNA Modification kit (Chemicon International Inc, Temecula, CA). Briefly, DNA (1 μg) was denaturated by adding NaOH (0.2 M) for 10 min at 50 °C. 550 μl of 3 M sodium bisulfite at pH 5.0 was added and mixed, and samples were incubated at 50 °C for 16 h in a water bath. Modified DNA was then bound to a micro-particulate carrier and was desalted by repeated centrifugation and resuspension in 70% ethanol. The conversion to uracil was completed by alkaline desulfonation, and DNA was finally eluted from the carrier by heating in TE buffer for 15 min at 60 °C. DNA preparations were either used immediately or stored at −20 °C.

Methylation-specific PCR—PCR analysis was performed as previously described (40). 2 μl of bisulfite-modified DNA was amplified by using PCR master mix (Promega Corp., Madison, WI), containing 25 units/ml of TaqDNA polymerase, 400 μM dNTPs, 1.5 mM $MgCl_2$, and 0.4 μM of each primer. The amplification program was as follow: 95 °C for 5 min; 35 cycles at 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, and a final extension at 72 °C for 5 min. The primers used for K10 amplification (M: methylation specific, U: specific for unmethylated sequence, W: unmodified specific) were the following: K10P MF (5′-AGTTTTCGTTTTCGTAGTCGTC-3′), K10P MR (5′-CGAATATAACCTCACCCCG-3′), K10P UF (5′-GGAGTTTTTGTTTTTGTAGTTGTT-3′), K10P UR (5′-AACCAAATATAACCTCACCCCA-3′), K10P WF (5′-AGCTTCCGCCTCCGTAGCCGCC-3′), and K10P WR (5′-CGAATGTGACCTCACCCCG-3′). PCR products were loaded on a 1.8% agarose gel containing ethidium bromide, and were visualized under UV illumination.

Genomic Methylation Level—A modification of the methyl-accepting assay (41) was used to determine the methylation level of DNA isolated from HaCaT cells. DNA (200 ng) was incubated with 4 units of SssI methylases (New England Biolabs, Ipswich, MA) in the presence of 1.5 mM *S*-adenosyl-L-[*methyl*-^{3}H]methionine and 1.5 mM nonradioactive *S*-adenosylmethionine (New England Biolabs). The reaction mixtures (20 μl), in the manufacturer's buffer containing 0.1 μg of RNase A, were incubated at 37 °C for 4 h. The reactions were terminated by adding 300 μl of stop solution (1% sodium dodecyl sulfate, 2 mM EDTA, 5% 2-propyl alcohol, 125 mM NaCl, 1 mg of proteinase K per ml, 0.25 mg of carrier DNA per ml) for 1 h at 37 °C. DNA was extracted with phenol-chloroform and was ethanol-precipitated. The recovered DNA was resuspended in 30 μl of 0.3 M NaOH and incubated for 30 min at 37 °C. DNA was spotted on Whatman GF/C filter discs, dried, and then washed five times with 5% (w/v) trichloroacetic acid followed by 70% (v/v) ethanol. Filters were placed in scintillation vials and incubated for 1 h at 60 °C with 500 μl of 0.5 M perchloric acid. Then, 5 ml of scintillation mixture was added, and the ^{3}H incorporation was determined by a Beckman liquid scintillation counter. Higher levels of [^{3}H]methyl group incorporated into DNA were indicative of lower levels of genomic DNA methylation (41).

Assay of DNA Methyltransferase—Cell extracts were prepared in ice-cold lysis buffer containing 50 mM Tris-HCl, pH 7.8, 1 mM EDTA, 10% glycerol, 0.01% sodium azide, 10% Tween-80, 100 μg/ml RNase A, and 0.5 mM phenylmethylsulfonyl fluoride. *De novo* methyltransferase activity was measured as previously described (42, 43). Cellular protein extracts (30 μg) were incubated in the presence of 3 μg of double-stranded oligonucleotides and 2.4 μCi of *S*-adenosyl-L-[*methyl*-^{3}H]methionine (Amersham Biosciences), at 37 °C for 1 h. The reaction was terminated by adding 90 μl of stop solution (1% sodium dodecyl sulfate, 2 mM EDTA, 3% (w/v) 4-amino salicylate, 5% butyl alcohol, 0.25 mg/ml calf thymus DNA, and 1 mg/ml proteinase K), and incubating at 37 °C for 45 min. The reaction mixture was then spotted on Whatman GF/C filter paper discs (Fisher Scientific, East Brunswick, NJ), and filters were washed twice with 5% trichloroacetic acid, rinsed in 70% ethanol, and dried at 56 °C for 20 min. Finally, filters were submerged in UltimaGold scintillation mixture (Packard, Meriden, CT) and radioactivity was measured in a Beckman liquid scintillation counter (LS 5000TD). A blank control reaction was done simultaneously using cell extracts that were heated to 80 °C for 15 min to inactivate the methyltransferase activity. The results, expressed as counts per min (cpm), were corrected by subtracting the background level.

Statistical Analysis—The data reported in this article are the mean ± S.D. of at least three independent determinations, each performed in duplicate. Statistical analysis was performed by the nonparametric Mann-Whitney *U* test, elaborating experimental data by means of the InStat 3 program (GraphPad Software for Science, San Diego, CA).

RESULTS

AEA Inhibits Keratinocyte Differentiation by Regulating Gene Expression—Spontaneously immortalized keratinocytes (HaCaT cells) can be induced to differentiate by treatment with TPA plus calcium (38, 44). Fig. 1 shows that, as expected, induction of differentiation of HaCaT cells for 5 days determines a significant increase in expression of genes known to be up-regulated during differentiation, as measured by quantitative RT-PCR. Notably, the increase in keratin 1 (K1), keratin 10 (K10), and transglutaminase 5 (TGase 5) (Fig. 1, *A*, *B*, *D*), which are all induced later during epidermal differentiation (38, 45, 46), was much higher than that of an early differentiation marker like involucrin (47) (Fig. 1*C*). Interestingly AEA treatment significantly reduced activation of differentiating genes. In addition, we observed that the mRNA level reduction (~50%) after AEA treatment was paralleled by a decreased protein level, at least in the case of K10 (Fig. 1*E*). These findings are well in line with our previous observation that AEA inhibits cornified envelope formation (24), and suggest that AEA is able to inhibit keratinocyte differentiation by modifying the gene expression profile of these cells.

Inhibition of DNA Methylation Prevents the Effects of AEA on Gene Expression—Because it has been shown that DNA methylation levels change during keratinocyte differentiation (32)

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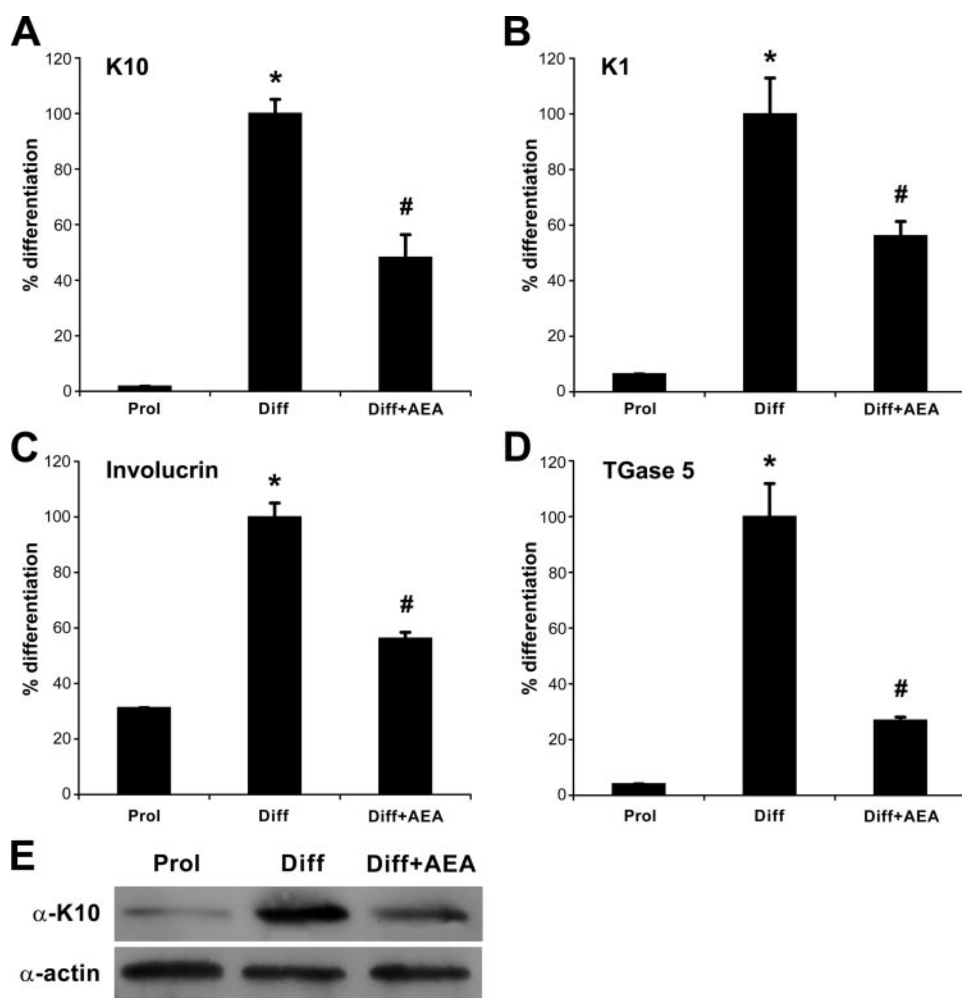

FIGURE 1. **AEA inhibits gene expression in differentiated keratinocytes.** *A–D*, differentiating HaCaT cells showed an increased expression of keratinocyte differentiation genes as compared with proliferating cells. Keratinocytes were induced to differentiate by treatment with TPA plus calcium for 5 days. Exposure to AEA (1 μM) induced a ~2-fold decrease of the expression of all tested genes, except for that of TGase 5, which decreased ~4-fold. K10, K1, involucrin and TGase 5 were detected by quantitative RT-PCR from differentiating and proliferating HaCaT cells. RT-PCR conditions and primers are detailed under "Experimental Procedures." For the quantitation of gene expression, β-actin was used as housekeeping gene. The amount of target genes, normalized to the housekeeping gene and relative to proliferating cells, was calculated by using the comparative C_T method. A validation experiment was performed, to demonstrate that efficiencies of target and housekeeping genes were approximately equal. The results are shown as percentage of gene expression (mean ± S.D.) relative to differentiating cells. Values represent the mean of at least four RT-PCR experiments, each performed in triplicate. *E*, Western blot analysis of proliferating, differentiating, and AEA-treated HaCaT cell extracts reacted with anti-K10 or anti-actin antibodies. AEA is able to significantly reduce K10 protein level in differentiating keratinocytes. *Prol*., proliferating cells; *Diff*., differentiating cells; *, $p < 0.01$ *versus* Prol.; #, $p < 0.01$ *versus* Diff.

and that inhibitors of methylation promote this phenomenon (33), we investigated the possibility that AEA was affecting gene expression levels through alteration of DNA methylation. Treatment of HaCaT cells with TPA plus calcium in the presence of 1 μM 5AC, an inhibitor of DNA methylation (48, 49), resulted in a ~2-fold increase in K10 expression, as compared with cells treated only with TPA plus calcium, suggesting that inhibition of DNA methylation allows increased transcription of this gene (Fig. 2). Most importantly, treatment with 1 μM 5AC abolished the effect of AEA on K10 expression levels, which were comparable to those of cells differentiated without AEA (Fig. 2). These data strongly suggest that inhibition of differentiation by AEA occurs through changes in chromatin methylation patterns, because inhibition of DNA methylation is sufficient to prevent AEA effects on keratinocyte differentiation.

AEA Decreases Gene Transcription by Inducing DNA Methylation—To validate the hypothesis that AEA could change DNA methylation levels in the K10 locus, we used a DNase I sensitivity assay, by which we tested nuclease accessibility in nuclei isolated from HaCaT cells. Proliferating cells exhibited marked resistance to increasing concentrations of DNase I compared with differentiating cells (Fig. 3*A*), where K10 gene was completely digested with one enzyme unit. As expected, treatment with 1 μM 5AC enhanced the sensitivity to DNase I treatment by reducing the methylation levels. Consistent with its possible role in regulating methylation levels, treatment of differentiating cells with AEA induced a strong resistance to DNase I digestion. Once again, 5AC was able to revert this effect, confirming a role for methylation in the activity of AEA on keratinocyte differentiation. To further confirm that the observed changes in K10 expression levels were due to changes in DNA methylation of the K10 locus, we directly examined the methylation status of K10 gene using a bisulfite based methylation-specific PCR (MSP) assay, which is sensitive and specific for methylation of any CpG located within a CpG island (40). The sequence differences resulting from bisulfite modification were shown by using primers that distinguish methylated from unmethylated DNA. Proliferating HaCaT cells showed a marked methylation status of K10 gene (Fig. 3*B*), consistent with low expression of this gene, while TPA plus calcium treatment resulted in decreased levels of methylated K10 and increased levels of the unmethylated form, again in line with the observed increase of expression levels. Additionally, AEA caused extensive methylation of the K10 gene in differentiating cells, and again treatment with 5AC determined the expected reduction of methylation regardless of the treatment with AEA. We next investigated whether AEA was able to induce DNA methylation beyond the K10 locus. To this end we measured the overall methylation levels in keratinocytes using an SssI methylase assay (Fig. 4). As reported in the literature, the genomic methylation levels decreased during keratinocyte differentiation (32). Treatment with AEA dramatically increased DNA methylation of differentiating cells, up to the lev-

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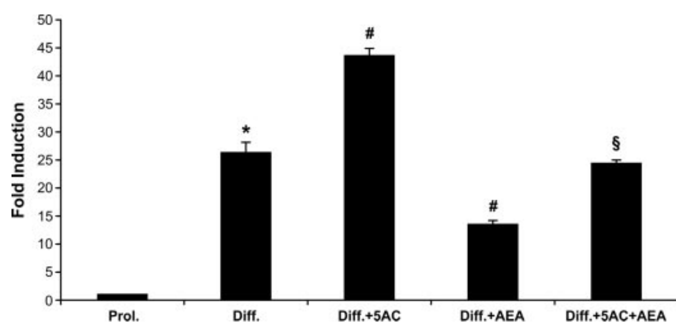

FIGURE 2. **AEA-mediated inhibition of keratinocyte differentiation is reverted by 5AC.** Differentiating HaCaT cells were treated with 1 μM AEA in the presence or in the absence of the methylation inhibitor 5AC (1 μM), and K10 gene quantitation was performed as described in the legend to Fig. 1. AEA treatment decreased K10 expression in differentiating cells, and this effect was reverted by treatment with 5AC. Note that treatment with the methylation inhibitor increased K10 expression, as compared with untreated differentiating cells, showing a direct correlation between the expression of this gene and genomic methylation. The results are shown as fold induction (mean ± S.D.) of four independent experiments. *Prol.*, proliferating cells; *Diff.*, differentiating cells; *, $p < 0.01$ *versus* Prol.; #, $p < 0.01$ *versus* Diff.; §, $p < 0.01$ *versus* Diff.+AEA.

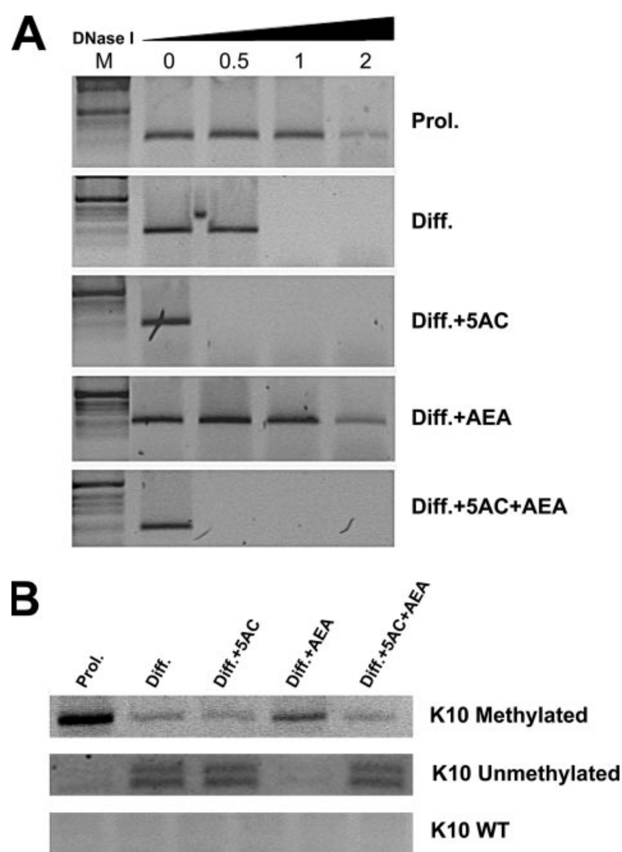

FIGURE 3. **AEA inhibits keratinocyte differentiation gene transcription by inducing DNA methylation.** *A*, DNase sensitivity assay. Nuclei isolated from proliferating and differentiating HaCaT cells, in the presence of AEA, 5AC, or both in a serum-free culture medium, were treated with 0, 0.5, 1, and 2 units of DNase I. Digested DNA was purified and used as template for PCR reactions. Primers were designed to amplify the K10 genomic region. PCR products were separated on 1.8% agarose gels and stained with ethidium bromide. The expected size for the K10 product is 208 bp. *B*, methylation-specific primed PCR. HaCaT cells were treated as above, and genomic DNA was extracted and modified by sodium bisulfite treatment, to convert unmethylated cytosine to uracil nucleotides, leaving unchanged methylated cytosine. In order to analyze the methylation status of K10 gene, specific primers for unmethylated or methylated sequences were used. To verify that all genomic DNA was modified by bisulfite treatment, a PCR reaction using non-methylation specific primers that are able to amplify only the unmodified DNA was performed. *M*, molecular weight marker; *Prol.*, proliferating cells; *Diff.*, differentiating cells; *WT*, not-methylation-specific primers.

els of proliferating cells (Fig. 4), thus confirming the general ability of AEA to modulate the methylation process.

AEA Induces DNA Methyltransferase Activity in Differentiating Keratinocytes—We next tested whether AEA affected genomic DNA methylation through regulation of DNA methyltransferase (DNMT) activity. Our results show that proliferating cells have much higher levels of methyltransferase activity compared with differentiating cells (Fig. 5). AEA treatment induced DNMT activity in differentiating cells, and this increase is likely to be sufficient to sustain the observed changes in DNA methylation. These data demonstrate that AEA can induce DNA methylation of keratinocyte-differentiating genes by increasing the activity of DNMT.

Role of CB1 Receptors and Other Endocannabinoids in Keratinocyte Differentiation and DNMT Activity—Finally, to further investigate the molecular details of the activity of AEA on epidermal differentiation, we evaluated the effect of SR141716, a selective CB1R antagonist (50), on the modulation of K10 expression and DNMT activity by AEA. It should be recalled that CB1R is the only cannabinoid receptor subtype expressed by HaCaT cells (24), that do not express the AEA-binding vanilloid receptor either (24). Treatment of differentiating keratinocytes with SR141716 was able to prevent AEA-mediated decrease of K10 expression (Table 1) and increase of DNMT activity (Fig. 5). Moreover 2-AG (51) and NADA (52), two endocannabinoids that are also able to activate CB1R, had the same effect as AEA on K10 mRNA (Table 1). Consistent with these findings, treatment of differentiating keratinocytes with ACEA, a selective CB1R agonist (53), led also to a significant decrease of K10 expression (Table 1) and increase of DNMT activity (Fig. 5).

CB1R activation by AEA and congeners triggers two common signaling pathways, that engage p38 and p42/p44 mitogen-activated protein kinases (MAPK) (54, 55). To further elucidate the molecular mechanism by which AEA affects epidermal differentiation, we ascertained the involvement of these MAPKs by using selective inhibitors at concentrations known to inhibit the target enzymes (56, 57). Our results show that SB203580, a selective inhibitor of p38 MAPK (56), was able to fully restore K10 expression in AEA-treated differentiating keratinocytes, whereas PD98059, a selective inhibitor of p42/p44 MAPK (57), had a smaller (yet significant) effect (Table 1). Moreover, treatment with SB203580 under the same experimental conditions was able to fully prevent also AEA-dependent increase of DNMT activity in differentiating keratinocytes (Fig. 5), further corroborating a key role for CB1R-dependent signaling in the activity of AEA during epidermal differentiation.

Altogether, these data suggest that the inhibition of epidermal differentiation by AEA was mediated by CB1R, ultimately leading to increased DNMT activity and increased genomic DNA methylation.

DISCUSSION

In this investigation we report evidence that the expression levels of several epidermal differentiation genes (*i.e.* keratins

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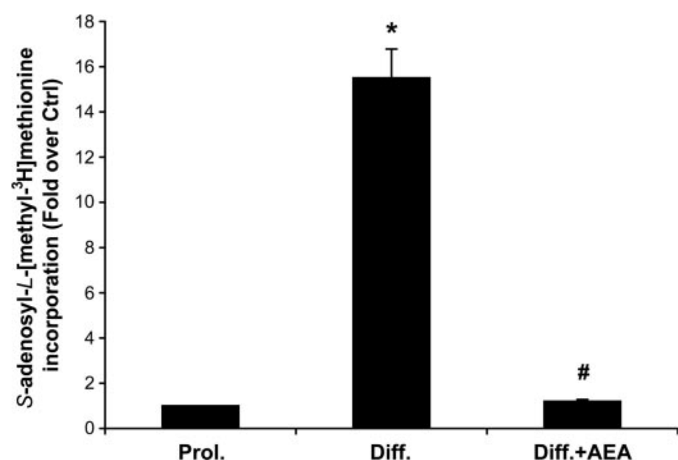

FIGURE 4. **AEA decreases genomic DNA methylation in differentiating keratinocytes.** Methylation levels of genomic DNA were measured from triplicate samples by a methyl-accepting assay with CpG methylase SssI, in the presence of *S*-adenosyl-L-[*methyl*-^{3}H]methionine (see "Experimental Procedures" for details). Higher levels of [^{3}H]methyl group incorporated into DNA indicated lower levels of genomic DNA methylation. CpG availability was normalized to proliferating cells and the relative methylation levels were calculated. *Prol.*, proliferating cells; *Diff.*, differentiating cells; *, $p < 0.01$ *versus* Prol.; #, $p < 0.01$ *versus* Diff.

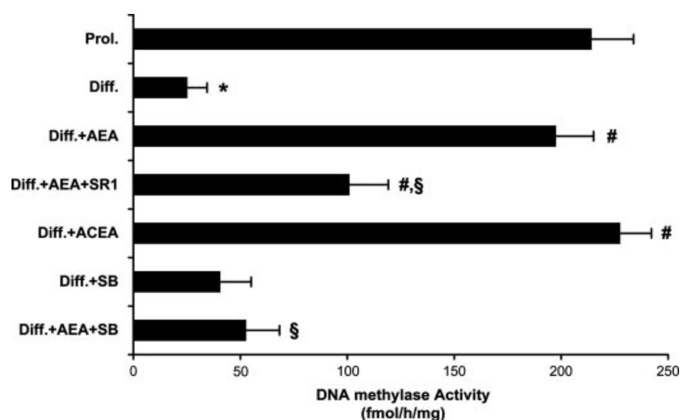

FIGURE 5. **AEA induces DNA methyltransferase activity in differentiated keratinocytes in a CB1-dependent manner.** Proliferating and differentiating keratinocytes treated with 1 μM AEA, 0.1 μM SR141716, 1 μM arachidonoyl-2-chloroethylamide, or 10 μM SB203580 were lysed, and DNA methyltransferase activity was measured as described under "Experimental Procedures." *Prol.*, proliferating cells; *Diff.*, differentiating cells; SR1, SR141716; ACEA, arachidonoyl-2-chloroethylamide; SB, SB203580; *, $p < 0.01$ *versus* Prol.; #, $p < 0.01$ *versus* Diff.; §, $p < 0.01$ *versus* Diff. + AEA.

and transglutaminases) are regulated by the endocannabinoid AEA. Moreover, we show that changes in gene expression induced by AEA are due to increased methylation of genomic DNA, and that the inhibition of methylation alone is sufficient to prevent this effect. In keeping with these findings we have previously shown that exogenous AEA is able to inhibit epidermal differentiation, by decreasing cornified envelope formation (24). Furthermore, we have previously shown that endogenous AEA levels in differentiating keratinocytes decrease, while the enzyme involved in its degradation (FAAH) increases, suggesting that the EC system might indeed play an important physiological role in regulating the differentiation process (24).

A role for methylation in the regulation of keratinocyte differentiation is not totally unexpected, because an inverse correlation between DNA methylation and the expression of differentiating genes has been identified in human keratinocytes (58, 59). It has been hypothesized that epigenetic mechanisms also participate in the control of genes located in the epidermal differentiation complex (EDC), on the chromosomal band 1q21 (58). This genomic region consists of multiple families of clustered genes that undergo coordinate regulation during keratinocyte differentiation (59). Even if K10 gene is not located in EDC, general changes in 5-methylcytosine contents were observed in keratinocytes during differentiation (32), suggesting that many different genetic loci are controlled through this mechanism during differentiation. The effect of AEA during keratinocyte differentiation is not restricted to K10 gene expression; indeed, we have demonstrated an overall increase of DNA methylation in differentiating keratinocytes treated with this endocannabinoid. Finally, we show that the action of AEA on gene expression and DNA methylation depends on CB1R and is not the result of a direct interaction between AEA and DNMT; accordingly, the effect of AEA is prevented by the CB1R antagonist SR141716, and is instead mimicked by other endocannabinoids that bind to CB1R, such as 2-AG (51) and NADA (52), and by the specific CB1R agonist ACEA (53). Moreover, we demonstrate that AEA-dependent effects on gene expression and DNMT activity require CB1R-mediated signaling, that engages p38 and (to a lesser extent) p42/44 MAPK activity. This is in line with our previous finding that the anti-differentiating effect of AEA is dependent on CB1R (24). Currently we know that AEA binding to CB1R inhibits adenylyl cyclase, voltage-gated L, N, and P/Q-type Ca^{2+} channels, while activating MAPK, K^+ channels, focal adhesion kinase and cytosolic phospholipase A_2 (4). However, as yet no information is available on the ability of endocannabinoids to regulate gene expression. We believe that the data reported in this investigation outline a new activity of endocannabinoids as transcriptional regulators. This observation can contribute to explain a number of recently described effects of endocannabinoids on cell proliferation and differentiation. In fact, besides inhibition of epidermal differentiation (24), activation of CB1R has been linked to neurogenesis and neural differentiation, and endocannabinoids have been shown to promote neural progenitor proliferation and astroglial differentiation, while inhibiting differ-

TABLE 1
Effect of CB1R antagonist SR141716 and AEA congeners on keratinocyte differentiation

Treatment	K10 expression (fold induction)
Prol.[a]	1.0 ± 0.3
Diff.[b]	49.1 ± 2.6[c]
Diff. + AEA (1 mM)	10.5 ± 0.1[c,d]
Diff. + SR141716 (0.1 mM)	52.1 ± 4.2[c]
Diff. + AEA (1 mM) + SR141716 (0.1 mM)	59.9 ± 16.6[c,e]
Diff. + 2AG (1 mM)	21.8 ± 0.7[c,d]
Diff. + NADA (1 mM)	21.7 ± 0.4[c,d]
Diff. + ACEA (1 mM)	8.7 ± 1.0[c,d]
Diff. + SB203580 (10 mM)	45.4 ± 11.2[c]
Diff. + AEA (1 mM) + SB203580 (10 mM)	38.6 ± 4.3[c,e]
Diff. + PD98059 (10 mM)	45.9 ± 5.3[c]
Diff. + AEA (1 mM) + PD98059 (10 mM)	20.6 ± 2.4[c,e]

[a] Prol., proliferating keratinocytes.
[b] Diff., differentiating keratinocytes.
[c] $p < 0.01$ *versus* Prol.
[d] $p < 0.01$ *versus* Diff.
[e] $p < 0.01$ *versus* Diff. + AEA.

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entiation of cortical neurons (37, 60). In mouse adipocytes, the expression of endocannabinoids reaches a peak before differentiation, and AEA is able *per se* to induce preadipocyte maturation (61). Additionally, the endocannabinoid system has been implicated in maintenance of bone mass, by controlling bone cells homeostasis (62) and in regulating human hair follicle growth (63), a process very close to epidermal differentiation. Up to date, the molecular details of these effects of AEA remain undisclosed. Also undisclosed remain the events underlying the effects of endocannabinoids on the expression of key S phase proteins in human breast cancer cells (64), or of genes regulating energy metabolism in human skeletal muscle (65). The transcriptional activity of endocannabinoids might represent a common mechanism to explain at once the above-mentioned effects of these compounds.

In conclusion, we believe that the importance of our findings goes beyond the role in keratinocyte differentiation that we have shown here. In fact, regulation of DNA methylation is a fundamental epigenetic modification of the genome that is involved in regulating a large number of cellular processes, including: embryonic development, transcription, chromatin structure, X chromosome inactivation, genomic imprinting, and chromosome stability. The importance of DNA methylation is also demonstrated by the growing number of diseases that occur when methylation is not properly established or maintained in cells (66). Among many other diseases, a role for altered methylation has been established in cancer. Cancer cells are usually hypomethylated and loss of genomic methylation is usually an early event in cancer development that also correlates with disease severity and metastatic potential (66). Genome-wide demethylation is usually accompanied by gene specific hypermethylation in cancer cells. Genes involved in apoptosis, cell cycle regulation, DNA repair, cell signaling, and transcription have been shown to be silenced by hypermethylation. There is therefore a growing interest in developing ways of pharmacologically reversing methylation abnormalities. We believe that our present observations might open the road to a number of studies that can potentially lead to the exploitation of endocannabinoid signaling to regulate DNA methylation in a variety of human pathologies. In this context, it seems noteworthy that stimulation of CB1R has been shown to inhibit *in vivo* ras oncogene-dependent tumor growth and metastasis (67). Moreover, in relation to human skin that also expresses CB1 receptors (68), during the preparation of this manuscript a cannabinoid antiproliferative action has been demonstrated on melanoma cells (69), that may contribute to design new therapeutic strategies for the management of this widespread skin cancer. In the same line, a protective role of the endocannabinoid system in contact allergy of the skin has been recently shown (70). Therefore, the finding that CB1R activation by AEA triggers DNA methylation in human keratinocytes could be relevant for the development of novel pharmacological treatments, able to reduce allergic inflammation through the promotion of epigenetic modifications.

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