The Endocannabinoid System in Human Keratinocytes

EVIDENCE THAT ANANDAMIDE INHIBITS EPIDERMAL DIFFERENTIATION THROUGH CB1-RECEPTOR-DEPENDENT INHIBITION OF PROTEIN KINASE C, ACTIVATING PROTEIN-1, AND TRANSGLUTAMINASE

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Anandamide (AEA), a prominent member of the endogenous ligands of cannabinoid receptors (endocannabinoids), is known to affect several functions of brain and peripheral tissues. A potential role for AEA in skin pathophysiology has been proposed, yet its molecular basis remains unknown. Here we report unprecedented evidence that spontaneously immortalized human keratinocytes (HaCaT) and normal human epidermal keratinocytes (NHEK) have the biochemical machinery to bind and metabolize AEA, i.e. a functional type-1 cannabinoid receptor (CB1R), a selective AEA membrane transporter (AMT), an AEA-degrading fatty acid amide hydrolase (FAAH), and an AEA-synthesizing phospholipase D (PLD). We show that, unlike CB1R and PLD, the activity of AMT and the activity and expression of FAAH increase while the endogenous levels of AEA decrease in HaCaT and NHEK cells induced to differentiate in vitro by 12-O-tetradecanoylphorbol 13-acetate (TPA) plus calcium. We also show that exogenous AEA inhibits the formation of cornified envelopes, a hallmark of keratinocyte differentiation, in HaCaT and NHEK cells treated with TPA plus calcium, through a CB1-dependent reduction of transglutaminase and protein kinase C activity. Moreover, transient expression in HaCaT cells of the chloramphenicol acetyltransferase reporter gene under control of the loricrin promoter, which contained a wild-type or mutated activating protein-1 (AP-1) site, showed that AEA inhibited AP-1 in a CB1-dependent manner. Taken together, these data demonstrate that human keratinocytes partake in the peripheral endocannabinoid system and show a novel signaling mechanism of CB1 receptors, which may have important implications in epidermal differentiation and skin development.

Endocannabinoids are amides, esters, and ethers of long-chain polyunsaturated fatty acids, found in several human tissues (1). Anandamide (N-arachidonoylethanolamine, AEA)1

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§ The abbreviations used are: AEA, anandamide (N-arachidonoylethanolamine); 2-AG, 2-arachidonoylglycerol; AM404, N-(4-hydroxyphenyl)arachidonoylamide; AMT, AEA membrane transporter; AP-1, activating protein-1; ATFMK, arachidonoyltriﬂuoromethylketone; CB1/2R, type 1/2 cannabinoid receptors; CP55,940, 5-(1,1'-dimethylheptyl)-2-[1(1R,5R)-hydroxy-2R-(3-hydroxypropyl)cyclohexy1]phenol; FAAH, fatty acid amide hydrolase; GAMBU-AP, goat anti-mouse (rabbit) antibodies conjugated to alkaline phosphatase; MAFF, methylarachidonoyl fluorophosphonate; NHEKs, normal human epidermal keratinocytes; PLD, phospholipase D; RT, reverse transcriptase; SIN-1, 3-morpholinosydnonimine; SNP, sodium nitroprusside; SR141716, N-(1-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-5-(methoxy-3-pyrazole-carboxamide); SR144528, N-[1(S)-endo-1,3,3-trimethylbicyclo[2.2.1]heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)pyrazole-3-carboxamide; TGFase, transglutaminase; NAPE, N-acylethanolamidase; PMA, phorbol 12-myristate 13-acetate; PMSF, phenylmethylsulfonyl fluoride; PKC, protein kinase C; CE, cornified envelope; CAT, chloramphenicol acetyltransferase; VR1, vanilloid receptor 1.
contain endogenous AEA, which mediates cellular response to ultraviolet B irradiation (16), and a stable analogue of AEA has been shown to inhibit the proliferation in vitro of rat epithelial cells, through a CB1-dependent mechanism (17). In addition, a recent report has shown that skin tumors of mice and men express CB1R and CB2R, that healthy human skin expresses CB1R, and that synthetic agonists of CBRs inhibit tumor growth (18). Moreover, isolated rat paw skin responds to an in vitro model of neuropathic pain according to a CB1-dependent mechanism (19). This background prompted us to investigate whether human keratinocytes have endogenous biochemical machinery to bind and metabolize AEA and how the endocannabinoid system might be implicated in the control of epidermal cell growth and differentiation.

EXPERIMENTAL PROCEDURES

Materials—Chemicals were of the purest analytical grade. Anandamide (AEA), 12-O-tetradecanoylphorbol 13-acetate (TPA), sodium nitroprusside (SNP), minimum essential medium, phenytoylsulfonyl fluoride (PMSF), N,N′-dimethylcysteine, and putrescine were purchased from Sigma Chemical Co. (St. Louis, MO). N-(4-Hydroxyphenyl)arachidonoylamine (AM404), arachidonyl trifluoromethylketone (ATFMK), and 2-arachidonylglycerol (2-AG) were from Research Biochemicals International (Natick, MA). 3-Morpholinosydnonimine (SIN-1) was from Alexis Corp. (Lauffelfingen, Switzerland). VDM11 was from Tocris Cookson (Bristol, UK), and methylarachidonoyl fluorophosphonate (MAFP) was from Cayman Chemicals (Ann Arbor, MI). Capsaiphen and the protein kinase C substrate myelin basic protein fragment 4-14 were from Calbiochem (La Jolla, CA). Cannabidiol was a kind gift of D. M. van der Stelt (Utrecht University, The Netherlands). N-Piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazolecarboxamide (SR141716) and N-[1(S)-endo-1,3,3-trimethylcyclo(2.2.1)-heptan-2-yl]-N-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)pyrazole-3-carboxamide (SR144528) were kind gifts of Prof. N. E. Fusenig (German Cancer Research Center, Heidelberg, Germany). [3H]Resiniferatoxin (48 Ci/mmol) was a kind gift of Dr. Vincenzo Di Marzo (Consiglio Nazionale delle Ricerche, Pozzuoli, Italy). N-[2-(4-Chlorophenyl)-5-(4-chlorophenyl)-3-(4-hydroxyphenyl)arachidamide (SR141716) and N-[1(S)-endo-1,3,3-trimethylcyclo(2.2.1)-heptan-2-yl]-N-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)pyrazole-3-carboxamide (SR144528) were kind gifts of Ms. van der Stelt (Utrecht University, The Netherlands). Cryopreserved nor-150, 0°C, 5% CO2 humidified atmosphere (22). Trypan Blue dye exclusion, as reported (24). Thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test and by nonradioactive analysis (also in this case, the uptake at 4°C was subtracted from that at 37°C). The effect of different compounds on the uptake (15 min) of 200 nM [3H]AEA by AMT was determined by adding each substance directly to the incubation medium, at the indicated concentrations. FAAH activity was assayed also in NHEK cells (2×106/test), using 400 nM [3H]AEA as substrate. Cell viability after each treatment was greater than 90% in all cases.

FAAH Activity and Expression—Fatty acid amide hydrolase (EC 3.5.1.4; FAAH) activity and its apparent Ki, Vmax, and Km values were determined in HaCaT cells (20 μg/test) as reported (26). FAAH activity was also assayed in NHEK cells (20 μg/test), using 5 μM [3H]AEA as substrate. HaCaT cell homogenates (20 μg/lane) were subjected to SDS-PAGE and blot analysis, as described (26). RT-PCR was performed using total RNA isolated from HaCaT cells (5×106 cells) by means of the SNAPool total RNA isolation kit (Invitrogen, Carlsbad, CA), as described (26). The primers were as follows: (−) 5′-TGGAGTCTCCTCCAAAGCCCG-3′, (−) 5′-TGTCATAGACAGGCCCTTCGA-3′, for FAAH; (−) 5′-AGTGGCTCAAGTTAACACCC-3′, (−) 5′-CCCTAGTTCCGAAAACCAAC-3′, for 18 S RNA.

Five microliters of the reaction mixture was electrophoresed on a 6% polyacrylamide gel, which was then dried and subjected to autoradiography (26). The autoradiographic films were subjected to densitometric analysis, by means of a Floor-STM Multi-Imager equipped with Quantity One software (Bio-Rad). The same anti-FAAH antibodies were used to determine FAAH protein content also by enzyme-linked immunosorbent assay of coating wells with FAAH homogenates (20 μg/test). FAAH activity was also assayed in NHEK cells (2×106/test) as means of the SNAPool total RNA isolation kit (Invitrogen, Carlsbad, CA), as described (26). The primers were as follows: (−) 5′-TTGAAGTTCTCCTCCAAAGCCCG-3′, (−) 5′-TGTCATAGACAGGCCCTTCGA-3′, for FAAH; (−) 5′-AGTGGCTCAAGTTAACACCC-3′, (−) 5′-CCCTAGTTCCGAAAACCAAC-3′, for 18 S RNA.

Other Biochemical Assays—The endocannabinoid levels of AEA in HaCaT and NHEK cells (5×106/test) were determined by gas chromatography-electron impact mass spectrometry, as recently reported (29). The activity of phospholipase D (EC 3.1.4.4; PLD) was assayed in homogenates of HaCaT and NHEK cells (50 μg/test) according to Meoosgaard et al. (30), using 1,2-dioleoyl-

Binding to Cannabinoid Receptors—For cannabinoid receptor studies, incubations were performed with HaCaT cells (2×105/test) as reported (24) and were used in rapid filtration assays with the synthetic cannabinoid [3H]CP55,940 (24). Apparent dissociation constant (Kd) and maximum binding (Bmax) values of [3H]CP55,940 were calculated from saturation curves through nonlinear regression analysis with the Prism 3 program (GraphPad, San Diego, CA) (24). CBR binding was assayed also in NHEK cells (25×105/test), using 200 μM [3H]CP55,940. Binding of [3H]AEA to HaCaT cells was evaluated with the same filtration assays used for [3H]CP55,940 (24), and apparent Kd and Bmax values were calculated through nonlinear regression analysis of saturation curves. Also binding of [3H]resiniferatoxin to HaCaT cells was evaluated by rapid filtration assays, performed as described previously (25). In all experiments, unspecific binding was determined in the presence of 10 μM unlabeled agonist (24, 25). The expression of CB1R and CB2R in HaCaT cells was assessed by Western blot analysis, performed as detailed below for FAAH, using anti-CB1 or anti-CB2 polyclonal antibodies (each diluted 1:250), and GAR-AP (diluted 1:2000) as second antibody (26). Saturation curves of [3H]CP55,940 binding and Western blot analysis of CB1R and CB2R were performed under the same experimental conditions on mouse brain and mouse spleen extracts.

Analysis of Anandamide Uptake—The uptake of [3H]AEA by the AEA membrane transporter (AMT) of intact HaCaT cells (2×106/test) was performed as described previously (27). To discriminate noncarrier-mediated from carrier-mediated transport of AEA through cell membranes, [3H]AEA uptake at 4°C was subtracted from that at 37°C (26). The Qmax value of AMT was calculated as the ratio of AEA uptake at 30°C and 20°C (28). Incubations (15 min) were also carried out with different concentrations of [3H]AEA, in the range 0–800 nM, to determine the apparent Michaelis-Menten constant (Km), maximum velocity (Vmax), and inhibition constant (Ki) of AMT by nonlinear regression analysis. Also in this case, cell uptake at 4°C was subtracted from that at 37°C. The Qmax value of AMT was determined by adding each substance directly to the incubation medium, at the indicated concentrations. FAAH activity was assayed also in NHEK cells (2×106/test), using 400 nM [3H]AEA as substrate. Cell viability after each treatment was greater than 90% in all cases.
HaCaT or NHEK cells (10^5/g/test) was determined by measuring the incorporation of [32P]ATP (5 Ci) into the highly selective substrate myelin basic protein fragment 4-14 (25 nM), as reported (22). Reactions were incubated for 10 min at 30°C, spotted onto Whatman P81 paper, and washed four times in 75 mM H3PO4. The bound radioactivity was determined by liquid scintillation counting in an LKB 1217 Rackbeta spectrometer.

Determination of Cornified Cell Envelopes—Cornified envelopes (CE) were extracted from HaCaT or NHEK cells (5 × 10^6/g/test) by exhaustive boiling and sonication in 2% SDS, 20 mM dithiothreitol, 0.1 mM Tris-HCl (pH 7.4) for 2 h at 95°C. The extracts were boiled for 5 min in 2% SDS, 20 mM dithiothreitol, 0.1 mM Tris-HCl (pH 7.4) and then centrifuged at 10,000 × g for 10 min. The supernatants were used for Western blot analysis.

Fig. 1. Cannabinoid receptors in human HaCaT cells. A, saturation curves of the binding of [3H]CP55,940 to mouse brain, mouse spleen, and HaCaT cells. Vertical bars represent ± S.D. values. B, displacement of 200 pmol [3H]CP55,940 by 1 µM AEA and by CB1 and CB2 receptor antagonists SR141716 and SR144528 (100% as in A). Vertical bars represent ± S.D. values. *, p < 0.01 versus controls (p > 0.05 in all other cases). C and D, Western blot analysis of mouse brain, mouse spleen, and HaCaT cell extracts (20 µg/lane), reacted with anti-CB1R (C) or anti-CB2R (D) polyclonal antibodies. Molecular mass markers are shown on the right.

HaCaT or NHEK cells (10 µg/test) was determined by measuring the incorporation of [γ-32P]ATP (5 µCi) into the highly selective substrate myelin basic protein fragment 4-14 (25 µM), as reported (22). Reactions were incubated for 10 min at 30°C, spotted onto Whatman P81 paper, and washed four times in 75 mM H3PO4. The bound radioactivity was determined by liquid scintillation counting in an LKB 1217 Rackbeta spectrometer.

Fig. 2. Anandamide (AEA) binding and degradation by human HaCaT cells. A, saturation curve of the binding of [3H]AEA to HaCaT cells. B, dependence of the AEA membrane transporter (AMT) activity in HaCaT cells on AEA concentration, at 37°C or at 4°C. C, dependence of the AEA hydrolase (FAAH) activity in HaCaT cells on AEA concentration, at pH 9.0 or at pH 5.0. In all panels, vertical bars represent ± S.D. values.
(pH 8.0), and 0.5 mM EDTA, as previously described (33). CE formation was quantified by spectrophotometry at 600 nm and was normalized to the protein content (33).

**Transient Transfections**—Transient transfections were performed in triplicate using Lipofectin (Invitrogen), according to the manufacturer’s instructions. HaCaT cells (1 × 10⁶/test) were transfected with both wild-type and AP-1 mutated minimal lacZin promoters, placed upstream of the chloramphenicol acetyltransferase (CAT) reporter gene, as described (34). Transfection efficiency was monitored by using a thymidine kinase β-galactosidase construct (Clontech, Palo Alto, CA). After transfection, cells were left untreated or were treated for 6 h with various amounts of AEA, alone or in the presence of 1 μM SR141716 or 1 μM SR144528. Cells were then harvested, and CAT activity was assayed using the CAT Enzyme Assay System (Promega, Madison, WI), according to the manufacturer’s protocol. CAT activities were normalized to protein content and β-galactosidase activity.

**Statistical Analysis**—Data reported herein are the mean (±S.D.) of at least three independent determinations, each in duplicate. Statistical analysis was performed by using the nonparametric Mann-Whitney test, elaborating experimental data by means of the InStat 3 program (GraphPad).

**RESULTS**

**The Endocannabinoid System in HaCaT Cells**—The synthetic cannabionoid [³H]CP55,940, which has high affinity to both CB1 and CB2 receptors (35), was bound dose-dependently to spontaneously immortalized HaCaT cells (Fig. 1A). These saturation curves were very close to those obtained with mouse brain membranes (Fig. 1A), a positive control for CB1R (35, 36), and allowed to calculate Kd values of 610 ± 79 and 598 ± 86 pm, and Bmax values of 1378 ± 81 and 1773 ± 115 fmol-mg protein⁻¹, for HaCaT cells and mouse brain, respectively. On the other hand, binding of [³H]CP55,940 to mouse spleen, a positive control for CB2R (35, 36), showed saturation curves (Fig. 1A) from which a Kd of 245 ± 32 pm and a Bmax of 277 ± 11 fmol-mg protein⁻¹ could be calculated. The Kd and Bmax values found here for mouse brain and spleen are in agreement with previous reports (reviewed in Ref. 35). Consistently with the binding data, 1 μM anandamide (AEA) and 0.1 μM SR141716, a selective CB1R antagonist (35), but not 0.1 μM SR144528, a selective CB2R antagonist (35), displaced [³H]CP55,940, suggesting that only CB1 receptors were expressed on HaCaT cell surface (Fig. 1B). To further confirm the presence of CB1 receptors, Western blot analysis of HaCaT cell extracts was performed and compared with mouse brain and spleen extracts. Western blot analysis showed that specific anti-CB1R, but not anti-CB2R, antibodies recognized a single immunoreactive protein of 50 kDa in NHEK cells, and, consistently, binding of 200 nM [³H]CP55,940, which has high affinity to rat forebrain CB1R (35), and, consistently, binding of 200 nM [³H]AEA to HaCaT cells was displaced by 1 μM SR141716 (down to 15% of the control value), but not by 1 μM SR144528 (85%) nor by both 1 μM cannabidiol, antagonist of the “endothelial-type” cannabinoid receptor (37), and 1 μM capsazepine, antagonist of vanilloid receptors (4) (~90% in each case). Consistent with these findings, HaCaT cells were unable to bind [³H]reserpin in concentrations up to 500 pm (bound radioactivity = 243 ± 72 dpm, compared with 200 ± 60 dpm of unspecific binding; p > 0.05), further suggesting that HaCaT cells are devoid of vanilloid receptors.

Intact HaCaT cells were able to accumulate [³H]AEA in a temperature- (Q₁₀ = 1.5), time- (t₁⁄₂ = 5 min), and concentration-dependent manner (not shown). [³H]AEA accumulation occurred according to a saturable process (Fig. 2B) typical of AMT (6, 27, 28), showing apparent Kd and Vmax values of 346 ± 42 nm and 125 ± 6 fmol-min⁻¹-mg protein⁻¹, respectively. The uptake of 200 nM [³H]AEA was almost completely inhibited by

**Table I. Endogenous levels of anandamide (AEA) in spontaneously transformed (HaCaT) and normal (NHEK) human keratinocytes, induced to differentiate by TPA plus calcium**

| Treatment | AEA content (pmol-mg protein⁻¹) |
|-----------|---------------------------------|
| In HaCaT cells | In NHEK cells |
| Control | 480 ± 90 (100%) | 375 ± 75 (100%) |
| TPA + Ca²⁺ (6 h) | 450 ± 90 (94%) | 360 ± 70 (96%) |
| TPA + Ca²⁺ (24 h) | 280 ± 55 (55%) | 200 ± 40 (53%) |
| TPA + Ca²⁺ (5 days) | 120 ± 25 (25%) | 95 ± 20 (25%) |

* p < 0.05.
* p < 0.01 versus controls (p > 0.05 in all other cases).
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Table II

| Parameter             | Control       | TPA + Ca<sup>2+</sup> (24 h) | TPA + Ca<sup>2+</sup> (5 days) |
|-----------------------|---------------|-------------------------------|-------------------------------|
| CB1R binding<sup>a</sup> | 350 ± 35 (100%) | 355 ± 35 (101%) | 365 ± 35 (104%) |
| AMT activity<sup>b</sup> | 60 ± 5 (100%)  | 95 ± 10 (185%<sup>f</sup>)  | 125 ± 12 (208%<sup>f</sup>) |
| FAAH activity<sup>c</sup> | 95 ± 10 (100%) | 165 ± 16 (174%<sup>f</sup>)  | 240 ± 24 (253%<sup>f</sup>) |
| PLD activity<sup>d</sup> | 70 ± 7 (100%)  | 72 ± 7 (103%) | 75 ± 6 (107%) |

<sup>a</sup> Expressed as fmol·mg<sup>-1</sup> protein (ligand: 200 pm [3H]CP55,940).
<sup>b</sup> Expressed as pmol·min<sup>-1</sup> ·mg protein<sup>-1</sup> (substrate: 400 nM [3H]AEA).
<sup>c</sup> <i>p</i> < 0.05 (<i>p</i> > 0.05 in all other cases).
<sup>d</sup> <i>p</i> < 0.01 versus controls (<i>p</i> > 0.05 in all other cases).
<sup>e</sup> Expressed as pmol·min<sup>-1</sup> ·mg protein<sup>-1</sup> (substrate: 5 µM [3H]AEA).
<sup>f</sup> Expressed as pmol·min<sup>-1</sup> ·mg protein<sup>-1</sup> (substrate: 10 µM 1,2-dioleoyl-3-phosphatidyl-[2-14C]ethanolamine).

10 µM AM404 (down to 20% of the control value) or 10 µM VDM11 (to 15% of the control), specific inhibitors of AMT (4, 38). Instead this uptake was doubled by the nitric oxide-donor SNP (5 mM) and by the peroxynitrite-donor SIN-1 (1 mM). Moreover, the uptake was inhibited by 50 µM PMSF (35% of the control), by 10 µM ATFMK (25%), or by 100 nm MAPPF (20%), selective inhibitors of FAAH activity (4, 7). Even 2-AG dose-dependently inhibited [3H]AEA uptake by AMT, acting as competitive inhibitor with an apparent <i>K<sub>i</sub></i> of 400 ± 50 nm.

HaCaT cells showed FAAH activity at pH 9.0, with apparent <i>K<sub>i</sub></i> of 12 ± 2 µM and <i>V<sub>max</sub></i> of 370 ± 27 pmol·min<sup>-1</sup> ·mg protein<sup>-1</sup> (Fig. 2C). The hydrolysis of [3H]AEA by HaCaT cells at pH 5.0 was hardly detectable (Fig. 2C), ruling out the involvement of the amidase described recently in lysosomes and mitochondria (7). On the other hand, [3H]AEA hydrolysis at pH 9.0 was fully inhibited by 50 µM PMSF (25% of the control), by 10 µM ATFMK (15%), or by 100 nm MAPPF (15%). Again, 2-AG dose-dependently inhibited [3H]AEA hydrolysis by FAAH, acting as competitive inhibitor with an apparent <i>K<sub>i</sub></i> of 8 ± 1 µM.

Changes in the Endocannabinoid System in Differentiating HaCaT Cells—Treatment of HaCaT cells with TPA plus calcium, typical inducers of keratinocyte differentiation (22, 33), led to a time-dependent increase in the activity of AMT and FAAH, reaching a statistically significant increase (~180–200% of the control values) after 24 h and a maximum (~250% after 5 days (Fig. 3A)). Western blot analysis of HaCaT cell extracts showed that specific anti-FAAH antibodies recognized a single immunoreactive band of the molecular size expected for FAAH, the intensity of which increased time-dependently upon treatment with TPA plus calcium (Fig. 3B). Densitometric analysis of the filter shown in Fig. 3B indicated that FAAH protein content in 5-day-treated cells increased to 260% with respect to the vehicle-treated cells (100% = 10,300 ± 1,500 units per mm<sup>2</sup>). On the other hand, 5-day-treated and control cells expressed the same levels of actin (Fig. 3B), ruling out that the different levels of FAAH in these cells might be due to different loading of proteins. The same anti-FAAH antibodies were used to quantify FAAH content by enzyme-linked immunosorbent assay, demonstrating that the increase of enzymic activity in HaCaT cells treated with TPA plus calcium (Fig. 3A) was paralleled by increased FAAH expression (not shown). RT-PCR amplification of HaCaT cell cDNA showed a single band of the expected molecular size for the FAAH gene, which was not affected by TPA plus calcium (Fig. 3C). In fact, densitometric analysis of the autoradiographic film shown in Fig. 3C indicated that in 5-day-treated cells FAAH mRNA was ~115% of that in untreated cells (100% = 7,200 ± 800 units per mm<sup>2</sup>). Under the same experimental conditions, the expression of the 18 S rRNA gene was also unaffected (Fig. 3C).

Unlike AMT and FAAH, CB1R binding was unaffected by treatment of HaCaT cells with TPA plus calcium and remained the same as in the controls in 5-day-treated cells (Fig. 3A). Moreover, HaCaT cells showed PLD activity, which was asayed under conditions found to be optimal for the N-acylphosphatidylethanolamines (NAPEs)-hydrolyzing PLD (30). A radiolabeled phosphatidylethanolamine was used instead of radiolabeled NAPEs, which are not commercially available (31). This is noteworthy, because NAPEs-hydrolyzing PLD activity is considered responsible for AEA synthesis, although the lack of specific inhibitors of this enzyme makes it difficult to further extend its analysis and to conclusively assess its contribution to AEA metabolism (8, 30, 31). At any rate, treatment of HaCaT cells with TPA plus calcium did not affect PLD activity, which remained the same as in the controls in 5-day-treated cells (Fig. 3A).

Treatment of HaCaT cells with TPA plus calcium time-dependently decreased the endogenous levels of AEA, reaching statistical significance after 24 h and a minimum (25% of the control value) after 5 days (Table I). Therefore, the levels of AEA inversely correlated with those of the AEA-degrading agents AMT and FAAH (compare Table I and Fig. 3A).

The Endocannabinoid System in Resting and Differentiating NHEK Cells—To further generalize the effects of AEA on human keratinocytes, the analysis of the endocannabinoid system was extended to normal human epidermal keratinocytes (NHEKs). These cells showed a full endocannabinoid system, i.e., CB1R, AMT, FAAH, and PLD (Table II). In particular, binding of 200 pm [3H]CP55,940 was displaced by 0.1 µM SR141716 (to ~15% of the controls) but not by 0.1 µM SR144528 (~95%), and anti-CB1R, but not anti-CB2R, antibodies recognized a single immunoreactive band in NHEK cell extracts (not shown). These data suggest that NHEK cells also express a functional CB1R, as does healthy human skin (18). NHEK cells were induced to differentiate under the same experimental conditions as HaCaT cells. Treatment with TPA plus calcium reduced AEA content in NHEK cells in a way quite analogous to that observed in HaCaT cells (Table I). In addition, TPA plus calcium modulated the endocannabinoid system in the same manner as that of HaCaT cells (Table II). Indeed, the activity of AMT and FAAH increased time-dependently, reaching ~200 and ~250% of the controls in 5-day-treated cells, whereas CB1R and PLD were not significantly affected (Table II).

Effect of Exogenous AEA on Keratinocyte Differentiation in Vitro—Treatment of HaCaT cells with TPA plus calcium led to a ~400% increase in cornified envelope (CE) formation (Fig. 4A), a hallmark of keratinocyte differentiation (22, 32, 33). The increase in CE formation was paralleled by increased activity of transglutaminase (TGase) (~330% of the controls) and protein kinase C (~250%), two enzymes strictly related to keratinocyte differentiation (22, 32, 33). Administration of AEA to HaCaT cells dose-dependently reduced CE formation, TGase activity, and PKC activity induced by TPA plus calcium, reaching a maximum effect at 1 µM (Fig. 4A). At this concentration, CE formation, TGase activity, and PKC activity were, respectively, only ~150, ~130, and ~120% of the vehicle-treated controls (Fig. 4A). The effect of 1 µM AEA was reversed by 0.1 µM SR141716, but not by 0.1 µM SR144528 (Fig. 4A), suggest-
FIG. 4. Effect of exogenous anandamide (AEA) on keratinocyte differentiation. A, effect of various amounts of AEA (0.1, 0.5, or 1 μM), alone or in the presence of 0.1 μM SR141716 or 0.1 μM SR144528, on the differentiation of HaCaT cells induced in vitro by TPA plus calcium. 100% = 0.050 ± 0.006 A590 units/mg protein⁻¹ (CE), 375 ± 35 pmol/h·mg protein⁻¹ (TGase), and 2000 ± 200 cpm/min·mg protein⁻¹ (PKC). B, CAT activity of HaCaT cells transfected with the AP-1-containing promoter. Cells were transiently transfected with CAT reporter vectors under the control of the wild-type (wt) or AP-1-mutated (mut) loricrin promoter. Transfected keratinocytes were left untreated or were treated for 6 h with various amounts of AEA (0.1, 0.5, or 1 μM), alone or in the presence of 0.1 μM SR141716 or 0.1 μM SR144528. CAT activity was normalized to β-galactosidase activity and protein content (see “Experimental Procedures”) and was expressed as the percentage of untreated controls, set to 100. In both panels, vertical bars represent ± S.D. values. In A: *p < 0.01; **p < 0.05 versus controls; n.p. < 0.01 versus TPA plus Ca²⁺; §p < 0.01 versus TPA plus Ca²⁺ plus AEA (1 μM) (p > 0.05 in all other cases). In B: *p < 0.01; **p < 0.05 versus controls; §p < 0.01 versus AEA (1 μM) (p > 0.05 in all other cases).

In this investigation we report evidence that human keratinocytes have a functional “endocannabinoid system,” i.e., AEA and the biochemical machinery to bind, synthesize, transport, and hydrolyze it. We also show that differentiating keratinocytes have decreased levels of endogenous AEA, due to increased degradation of this lipid through AMT and FAAH. In addition, we demonstrate that exogenous AEA inhibits keratinocyte differentiation in vitro, through a CB1-dependent mechanism that involves inactivation of protein kinase C, activating protein-1, and transglutaminase.

Spontaneously immortalized HaCaT cells express functional type-1 cannabinoid receptors on their surface, as suggested by: (i) the Kᵢᵦ and Bmax values calculated from saturation binding curves (Figs. 1A and 2A); (ii) the displacement of [125I]CP55,940 (Fig. 1B) and of [3H]AEA by SR141716, and (iii) the cross-reactivity with specific anti-CB1R antibodies (Fig. 1C). The level of CB1R is constant in differentiating keratinocytes, whereas FAAH activity increases time-dependently (Fig. 3A), due to a higher gene expression at translational level (Fig. 3B). The uptake of AEA through its specific carrier (AMT) also increases in differentiating HaCaT cells (Fig. 3A). However, the molecular properties of AMT are not known, and no probes are available to measure its expression (6). AMT activity was fully prevented by treatment of HaCaT cells with FAAH inhibitors ATMFK and MAFF, in keeping with a facilitated transport driven by FAAH (40, 41). It is important to recall that the role of AMT in AEA degradation is still under debate, because FAAH might not need a transporter at all to become in contact with AEA (42), whereas AMT might work “in reverse” to export (rather than import) AEA (43). Recently even the existence of AMT has been questioned, suggesting that AEA is transported by an FAAH-driven simple diffusion process (44). Yet, recent investigations, showing that FAAH, but not AMT, is activated by progesterone (26), that AMT, but not FAAH, is activated by nitric oxide and peroxynitrite (27), and that estrogen activates AMT while inhibiting FAAH (43), seem to favor the existence of an AEA transporter distinct from the AEA hydrolase. A similar hypothesis is suggested by different human pathologic conditions, where FAAH, but not AMT, is modulated (reviewed in Ref. 1). At any rate, the apparent affinity of HaCaT AMT for AEA is very close to that of human lymphocytes (26) and of human endothelial cells (27), suggesting that the same carrier might be present on the surface of different peripheral cells. Moreover, AEA uptake by HaCaT AMT, like that of other human peripheral cells, was significantly increased by the nitric oxide donor SNP and even more by the peroxynitrite donor SIN-1 (24, 27). Unlike AMT and FAAH, PLD was not affected

and Fig. 4A). Importantly, AEA-induced reduction of CE formation in differentiating HaCaT and NHEK cells was not associated with a decrease in viability, which remained >90% of the cells treated with TPA plus calcium in both cases.

To further investigate the effect of AEA on the differentiation of human keratinocytes, transient transfection studies were performed in HaCaT cells with a vector containing the CAT gene under the control of the loricrin promoter, which contains activating protein-1 (AP-1)-responsive sites (22, 39). Incubation with AEA dose-dependently decreased CAT activity, down to 40% of vehicle-treated controls at 1 μM (Fig. 4B). SR141716, but not SR144528 (each used at 0.1 μM), fully prevented the effect of 1 μM AEA (Fig. 4B). Moreover, the AEA-induced inhibition of CAT activity of the wild-type loricrin promoter was completely abolished by its counterpart containing an AP-1-mutated site (Fig. 4B). These data suggest that the effect of AEA on the promoter was CB1-dependent and required an intact AP-1 element.

DISCUSSION

In this investigation we report evidence that human keratinocytes have a functional "endocannabinoid system," i.e., AEA and the biochemical machinery to bind, synthesize, transport, and hydrolyze it. We also show that differentiating keratinocytes have decreased levels of endogenous AEA, due to increased degradation of this lipid through AMT and FAAH. In addition, we demonstrate that exogenous AEA inhibits keratinocyte differentiation in vitro, through a CB1-dependent mechanism that involves inactivation of protein kinase C, activating protein-1, and transglutaminase.
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**TABLE III**

| Parameter                  | Control                  | TPA + Ca\textsuperscript{2+} | TPA + Ca\textsuperscript{2+} + AEA (1 μM) |
|----------------------------|--------------------------|-------------------------------|------------------------------------------|
| CE formation\textsuperscript{*} | 0.080 ± 0.007 (100%)    | 0.405 ± 0.040 (506%)\textsuperscript{b} | 0.115 ± 0.015 (144%)\textsuperscript{c,d} |
| TGase activity\textsuperscript{a} | 435 ± 40 (100%)         | 1745 ± 170 (401%)\textsuperscript{b} | 530 ± 50 (122%)\textsuperscript{d}       |
| PKC activity\textsuperscript{a}  | 2500 ± 240 (100%)       | 7500 ± 680 (300%)\textsuperscript{b} | 2650 ± 250 (118%)\textsuperscript{d}     |

\textsuperscript{*} Expressed as A\textsubscript{100} units/mg protein\textsuperscript{−1}.

\textsuperscript{a} p < 0.01.

\textsuperscript{b} p < 0.05 versus controls.

\textsuperscript{c} p < 0.01 versus TPA + calcium (p > 0.05 in all other cases).

\textsuperscript{d} Expressed as pmol/min/mg protein\textsuperscript{−1}.

\textsuperscript{e} Expressed as cpm/min/mg protein\textsuperscript{−1}.

by treatment of HaCaT cells with TPA plus calcium, suggesting that the decreased AEA content in differentiating keratinocytes was due to a greater degradation only. A critical role of FAAH in controlling endogenous levels of AEA in human keratinocytes is in keeping with the hypothesis that FAAH is the key regulator of AEA levels in vivo (42); indeed, FAAH knockout mice show ~15-fold higher levels of AEA than wild-type littermates (45), and AEA levels in human blood inversely correlate with FAAH activity in peripheral lymphocytes (1). Incidentally, the levels of AEA in untreated human keratinocytes reported in Table I correspond to ~3 pmol/mg of lipid phosphorus, a value comparable to that reported in mouse epidermal cells (16).

The up-regulation of AMT and FAAH upon keratinocyte differentiation, and the subsequent down-regulation of intracellular AEA, are major findings of this investigation, which were further generalized to normal human epidermal keratinocytes (Tables I and II). Accordingly, exogenous AEA was able to inhibit the formation of cornified envelopes induced in vitro by TPA plus calcium (Fig. 4A). Many biochemical markers of differentiation can be identified in keratinocytes, namely keratin, involucrin, filaggrin, and loricrin. All these differentiation products are required for the assembly of CE, a specialized structure that provides a barrier for the organism (46, 47). Therefore, CE formation is widely used as a specific hallmark of keratinocyte differentiation (22, 32, 46, 47). Here we show that AEA inhibited CE formation in differentiating human keratinocytes (Fig. 4A). Consistently, AEA reduced the activity of transglutaminase, which increases in differentiating cells (Fig. 4A; see also Ref. 48), where it catalyzes cross-linking reactions required for the orderly assembly of the CE (23).

In addition, in differentiating keratinocytes AEA reduced the activity of PKC, which also has been demonstrated to be essential for epidermal differentiation (22). The findings on CE formation and on the activity of TGase and PKC were extended to normal human epidermal keratinocytes, suggesting that they may be physiologically relevant also in vivo (Table III). It seems noteworthy that PKC is an essential upstream regulator of AP-1 (Fig. 4), a nuclear transcription factor playing a major role in epidermal differentiation (34, 39). TGase 1, which is expressed in human keratinocytes (23), is responsive to AP-1 (32). AEA inhibited transient expression of CAT reporter gene under control of an intact AP-1 (Fig. 4B), consistent with the inhibition of PKC (Fig. 4A). Taken together, it can be suggested that AEA, by inactivating AP-1 and its obligatory activator PKC, may reduce TGase expression, thus inhibiting CE formation.

The anti-differentiating effect of AEA did not imply a reduced viability of differentiating keratinocytes, suggesting that it was not due to induction of cell death. This seems of interest, also in the light of a report on the ability of AEA to inhibit differentiation of rat neuronal cells, which appeared during the preparation of this report (50). Like in human keratinocytes, AEA-induced reduction of neuronal differentiation was not associated with a decrease in cell viability and was mediated by CB1 receptors (50). Taken together, these studies suggest that the anti-differentiating effect of AEA may be more generally involved in neuronal and epidermal development. Finally, the observation that binding of AEA to CB1 receptors inhibits PKC in differentiating HaCaT and NHEK cells (Fig. 4A and Table III) uncovers a novel mechanism of CB1R-dependent signal transduction (51, 52). Keeping in mind that PKC is required for vanilloid receptor 1 (VR1) activation (53), whereas it causes CB1R down-regulation (54), a dual regulation of PKC by VR1 or CB1R might have implications in the control of cell survival and death by AEA (11). In fact, activation of these receptors by AEA leads to opposite effects: anti-apoptotic, for CB1R, or pro-apoptotic, for VR1 (24, 55). However, further studies are necessary to assess the contribution of receptor-mediated PKA signaling to the biological actions of AEA.

In conclusion, we report evidence that human keratinocytes have a functional “endocannabinoid system,” which may sustain the peripheral actions of AEA at the skin level. Our findings give a biochemical foundation for the effects of AEA on epidermal cells, especially in relation to pain sensation (12), response to UV irradiation (16), cell proliferation (17), and tumor growth (18). In this context, the finding, that human keratinocytes partake in the peripheral endocannabinoid system and that AEA can inhibit epidermal differentiation, opens new perspectives in the understanding of skin development and in the treatment of human skin diseases where cell hyper-proliferation takes place.

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