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Suppression of Nerve Growth Factor Trk Receptors and Prolactin Receptors by Endocannabinoids Leads to Inhibition of Human Breast and Prostate Cancer Cell Proliferation*

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ABSTRACT

Anandamide and 2-arachidonoylglycerol (2-AG), two endogenous ligands of the CB1 and CB2 cannabinoid receptor subtypes, inhibit the proliferation of PRL-responsive human breast cancer cells (HBCCs) through down-regulation of the long form of the PRL receptor (PRLr). Here we report that 1) anandamide and 2-AG inhibit the nerve growth factor (NGF)-induced proliferation of HBCCs through suppression of the levels of NGF Trk receptors; 2) inhibition of PRLr levels results in inhibition of the proliferation of other PRL-responsive cells, the prostate cancer DU-145 cell line; and 3) CB1-like cannabinoid receptors are expressed in HBCCs and DU-145 cells and mediate the inhibition of cell proliferation and Trk/PRLr expression. β-NGF-induced HBCC proliferation was potently inhibited (IC_{50} = 50–600 nM) by the synthetic cannabinoid HU-210, 2-AG, anandamide, and its metabolically stable analogs, but not by the anandamide congener, palmitoylethanolamide, or the selective agonist of CB2 cannabinoid receptors, BML-190. The effect of anandamide was blocked by the CB1 receptor antagonist, SR141716A, but not by the CB2 receptor antagonist, SR144528. Anandamide and HU-210 exerted a strong inhibition of the levels of NGF Trk receptors as detected by Western immunoblotting; this effect was reversed by SR141716A. When induced by exogenous PRL, the proliferation of prostate DU-145 cells was potently inhibited (IC_{50} = 100–300 nM) by anandamide, 2-AG, and HU-210. Anandamide also down-regulated the levels of PRLr in DU-145 cells. SR141716A attenuated these two effects of anandamide. HBCCs and DU-145 cells were shown to contain 1) transcripts for CB1 and, to a lesser extent, CB2 cannabinoid receptors, 2) specific binding sites for [^{3}H]SR141716A that could be displaced by anandamide, and 3) a CB1 receptor-immunoreactive protein. These findings suggest that endogenous cannabinoids and CB1 receptor agonists are potential negative effectors of PRL- and NGF-induced biological responses, at least in some cancer cells. (*Endocrinology* **141:** 118–126, 2000)

TWO RECEPTOR subtypes for marijuana's psychoactive component, (−)-Δ^9-tetrahydrocannabinol, one, named CB1, most abundant in the brain and some peripheral tissues, and the other, named CB2, almost exclusively expressed in immune cells, have been characterized to date (see Ref. 1 for review). These findings together with the discovery of two endogenous ligands for these receptors, *N*-arachidonoylethanolamine (anandamide) (2) and 2-arachidonoylglycerol (2-AG) (3, 4), named endocannabinoids (ECs), led to the suggestion of the existence in mammals of an endogenous cannabinoid system. The metabolism and pharmacological properties of the ECs have been thoroughly investigated (see Refs. 5–10 for recent reviews), and yet the physiological role of these metabolites is still a matter for speculation. Anandamide and/or 2-AG were suggested to participate in several physiopathological situations, ranging from the modulation of neuronal (6) and immune cell (7) function to neuroprotection (8), control of cardiovascular and endocrine functions (9, 10), and antinociception (11, 12). We recently reported that anandamide arrests selectively the proliferation of human breast cancer cells (HBCCs) by inhibiting DNA synthesis and the G_1/S transition of the cell cycle (13). This effect is exerted through the activation of as yet unidentified cannabinoid-binding sites and via the down-modulation of the expression of the long form of the receptor (PRLr) of PRL, one of the endogenous hormones necessary to these cells to proliferate and differentiate (14). We also reported that anandamide together with its putative phospholipid biosynthetic precursor (5, 6, 15) and the amidohydrolase responsible for anandamide degradation (for a review, see Ref. 16) are present in HBCCs. *Cis*-9-octadecenoamide, a bioactive compound that inhibits anandamide hydrolysis (5), also exerts a weak antimitogenic action, probably by raising the levels of endogenous anandamide in HBCCs (17). These data suggest

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that anandamide may function as a local down-modulator of HBCC proliferation. The present study was aimed at providing answers to questions raised by our previous investigations. Are ECs also capable of inhibiting the mitogenic action induced by other growth factors? Is the proliferation of other PRL-responsive cancer cells also inhibited by these endogenous mediators? Is the antiproliferative effect of ECs mediated by the CB1 or the CB2 subtype of cannabinoid receptors? We have investigated whether and through what mechanism anandamide and 2-AG also inhibit the nerve growth factor (NGF)-induced proliferation of HBCCs and the PRL-induced proliferation of human prostate cancer cells.

Materials and Methods

Materials

Mammary carcinoma F-7 (MCF-7), human breast T-47D cancer cells, and human prostate DU-145 cancer cells were purchased from American Type Culture Collection (Manassas, VA) and cultured as advised by the manufacturer. Anandamide and palmitoylethanolamide were synthesized as described previously (2). Arvanil [*N*-(3-methoxy-4-hydroxybenzyl)-arachidonoylamide], a hybrid agonist at CB1 and vanilloid receptors, was synthesized as described previously (17a). 2-AG and the synthetic cannabinoid HU-210 were donated by Prof. R. Mechoulam, Hebrew University of Jerusalem (Jerusalem, Israel). SR141716A and SR144528 were gifts from Sanofi Pharmaceuticals, Inc. (Montpellier, France). (*R*)-Methanandamide and BML-190 were purchased from Biomol (Plymouth Meeting, PA), capsazepine was obtained from Alexis Corp. (San Diego, CA), and human PRL and recombinant β-NGF were purchased from Sigma. [^{3}H]SR141716A (55 Ci/mmol) was purchased from Amersham Pharmacia Biotech (Aylesbury, UK).

RT-PCR amplification of CB1/CB2 messenger RNA (mRNA)

Total RNA was prepared from MCF-7, T-47D, and DU-145 cells by the acid guanidium thiocyanate-phenol-chloroform method. Complementary DNA (cDNA) synthesis was performed in a 20-μl reaction mixture containing 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM deoxy-NTPs, 50 mM Tris-HCl (pH 8.3), 5 μg total RNA, 20 U ribonuclease inhibitor (Roche Molecular Biochemicals, Indianapolis, IN), 0.125 A_{260} units hexanucleotide mixture (Roche Molecular Biochemicals) for random priming, and 200 U Moloney murine leukemia virus reverse transcriptase (Superscript, Life Technologies, Inc., Gaithersburg, MD). The cDNA reaction mixture was incubated at room temperature for 10 min and at 37 C for 90 min, and the reaction stopped by heating at 98 C for 5 min followed by lowering the temperature to 4 C. RT-PCR amplification was performed using 0.5–3 μl cDNA reaction mixture and 2 U *Thermus icelandicus* DNA polymerase (Red-Hot, Advanced Biotechnologies, Columbia, MD) in a 25-μl PCR reaction mixture containing 25 mM Tris-[hydroxymethyl]-methyl-propane-sulfonic acid, sodium salt buffer (pH 9.3), 50 mM KCl, 1.5 mM $MgCl_2$, 1 mM β-mercaptoethanol, 12% dimethylsulfoxide, 250 μM of each deoxy-NTP, and 0.5 μM each of 5′- and 3′-primers. Reactions were performed in a PE Gene Amp PCR System 9600 thermocycler (Perkin-Elmer Corp., Norwalk, CT). The amplification protocol consisted of an initial denaturation of 5 min at 95 C, followed by 25–40 cycles of 1 min at 95 C, 1 min at 55 C, and 2 min at 75 C. A final cycle of 10 min was carried out at 75 C. The primers used were: CB1 sense primer, 5′-CGC AAA GAT AGC CGC AAC GTG T-3′; CB1 antisense primer, 5′-CAG ATT GCA GTT TCT CGC AGT T-3′; CB2 sense primer, 5′-TTT CCC ACT GAT CCC CAA TG-3′; CB2 antisense primer, 5′-AGT TGA TGA GGC ACA GCA TG-3′; β_2-microglobulin sense primer, 5′-CCA GCA GAG AAT GGA AAG TC-3′; and β_2-microglobulin antisense primer, 5′-GAT GCT GCT TAC ATG TCT CG-3′. The expected sizes of the amplicons were 244 bp for CB1, 337 bp for CB2, and 268 bp for β_2-microglobulin. The β_2-microglobulin housekeeping gene expression was used to evaluate any variation in the RNA content and cDNA synthesis in the different preparations. Furthermore, the PCR primers for β_2-microglobulin were selected on the basis of the sequence of the β_2-microglobulin gene (NCBI accession no. M17987) by including the intron 402-1017. These primers, in the presence of contaminant DNA, would have generated an amplicon of the expected size of 886 bp. Ten microliters of the PCR products were analyzed by electrophoresis on 2% agarose gels (MS agarose, Roche Molecular Biochemicals) in 1 × Tris-acetate-EDTA buffer at 4 V/cm for 4 h. Ethidium bromide (0.1 μg/ml) was included in both the gel and the electrophoresis buffer, and the PCR products were detected by exposure under UV light. No PCR product was detected in the absence of cDNA, primers, or Red-Hot DNA polymerase.

Binding assays

Binding assays in MCF-7, T-47D, and DU-145 cells were carried out using the filtration procedure reported previously (13), and membranes were prepared as therein described, except for the absence of phenylmethylsulfonylfluoride from the binding buffer. The binding of increasing concentrations (100–10000 pM) of [^{3}H]SR141716A to aliquots (0.4 mg total proteins) of these membranes and the displacement of a fixed concentration (300 pM) of [^{3}H]SR141716A by increasing concentrations (0.025, 0.1, 0.5, 1.0, and 5.0 μM) of anandamide or arvanil were measured in equilibrium assays. SR141716A (10 μM) was used to determine nonspecific binding. Receptor binding results were analyzed with GraphPad software (GraphPad Software, Inc., San Diego, CA). Scatchard curves for the binding of [^{3}H]SR141716A were used to calculate the binding capacity (B_{max}) and K_d for this ligand using nonlinear regression, and one- and two-site analyses were compared to determine better fit values (r^2 = 0.88 for one-site binding). Displacement curves (calculated by means of Pharm/PCS software [Microcomputer Specialists (MCS), Philadelphia, PA)] were used to calculate the K_i values for anandamide by inserting the corresponding IC_{50} values from the best-fitting curves into the Cheng-Prusoff equation.

Cell proliferation assays

Cell proliferation assays were carried out according to the method previously described (13) in 6-well dishes containing subconfluent cells (at a density of about 50,000 cells/well). With MCF-7 and T-47D cells test substances were introduced 3 h after cell seeding and then daily at each change of medium. Cells were treated with trypsin and counted by a hemocytometer 4 days after the addition of test substances. No significant decrease in cell viability (as assessed by trypan blue) was observed with up to 100 μM anandamide. With DU-145 cells, we used a previously described procedure (18). Substances were added 3 h after cell seeding (50,000 cells/well). PRL (1 mIU/ml) or vehicle was then added after 24 h with the change of medium, in the presence of the test substances or vehicle. After 72 h, cells were treated with trypsin and counted by a hemocytometer. To study the effect of NGF on MCF-7 cell proliferation, we used the previously described procedure (19). Twenty-four hours after cell seeding (50,000 cells/well), the medium was changed to serum-free medium, and cells were starved for 24 h. Cells were then treated with serum-free medium containing β-NGF (100 ng/ml) plus test substances or vehicle and treated with trypsin, then counted after 48 h. Means were compared by means of unpaired Student's *t* test, using $P < 0.05$ as the threshold for statistical significance.

Western immunoblotting

Cells in 100-mm petri dishes were treated with test substances under the same conditions those described above for cell proliferation assays. Cells were then washed twice with 137 mM NaCl, 3 mM KCl, 12 mM Na_2HPO_4, and 2 mM KH_2PO_4 (pH 7.4) and lysed with a lysis buffer consisting of 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 150 mM NaCl, 1 mM Na_3VO_4, 1 mM NaF, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM phenylmethylsulfonylfluoride, 1% Triton X-100 and 1 μg/ml each of aprotinin, leupeptin, and pepstatin A. Lysates were loaded onto gels containing, respectively, 10% and 7.5% polyacrylamide for blotting of PRLr and CB1 or Trk, respectively. Proteins were transferred to nitrocellulose membranes, which were then incubated first for 1 h at room temperature with the first antibody, *i.e.* either antihuman PRL receptor monoclonal antibody (U5, purchased from Affinity BioReagents, Inc., Golden, CO; 1:1000), antimouse Trk monoclonal antibody (B-3, Santa

Cruz Biotechnology, Inc., Santa Cruz, CA; 1:500), or CB1 polyclonal antibody (Cayman, Ann Arbor, MI; 1:800) and then with the appropriate horseradish peroxidase-labeled second antibody conjugates (1:5000; Bio-Rad Laboratories, Inc., Hercules, CA). Bands were visualized by the enhanced chemiluminescence technique (Bio-Rad Laboratories, Inc.). The anti-Trk and CB1 antibodies cross-react with human Trk and CB1.

Results

Effects of selective CB1 and CB2 agonists and antagonists on basal HBCC proliferation

The molecular targets for the action of each of the substances used in this study are listed in Table 1. The effects of anandamide, the synthetic cannabinoid HU-210, the metabolically stable anandamide analog (*R*)-methanandamide, 2-AG, and the anandamide congener, palmitoylethanolamide, on human EFM-19 breast cancer cells and of anandamide on MCF-7 and T-47D cells have been described previously (13). As shown in Table 2, the IC_{50} values for the antiproliferative effect of anandamide on MCF-7 and T-47D cells, after only a few subculturing passages, were 1.4 and 1.9 μM, respectively. IC_{50} values as high as 7 μM were instead found for anandamide when using MCF-7 cells that had undergone between 10–15 subculturing passages. 2-AG was less active than anandamide in T-47D, but not in MCF-7 (Table 2) or EFM-19 (13), cells. As shown in Fig. 1A, the selective CB2 agonist BML-190 (20) did not exert any antiproliferative action on HBCCs up to a 10-μM concentration, whereas arvanil, a CB1 agonist (see below), was a potent inhibitor of HBCC proliferation. The selective CB2 antagonist SR144528 (21) did not reverse the antiproliferative action of anandamide and arvanil, whereas the CB1 antagonist SR141716A did, in agreement with our previous report (13). The vanilloid receptor antagonist, capsazepine (not shown), also counteracted the effect of arvanil, in agreement with the capability of this compound to activate both CB1 and vanilloid receptors, whose presence in HBCCs was recently suggested (17a).

Endogenous and synthetic cannabimimetics inhibit β-NGF-induced MCF-7 cell proliferation

In agreement with a previous study (19), starved MCF-7 cells start proliferating after treatment with 100 ng/ml human β-NGF. A 91 ± 13% (n = 16) increased proliferation was observed *vs.* that of vehicle-treated cells. This process was blocked by submicromolar doses of anandamide, 2-AG, (*R*)-methanandamide, the synthetic cannabinoid HU-210, and arvanil, but not by BML-190 or palmitoylethanolamide (Fig. 1B). The antiproliferative effects of anandamide and arvanil were attenuated by SR141716A, but not by SR144528 (Fig. 1C). Capsazepine also counteracted the effect of arvanil (data not shown).

Endogenous and synthetic cannabimimetics suppress Trk levels in MCF-7 cells

MCF-7 cells express both low (p75) and high (Trk) affinity NGF receptors (19). We detected the latter receptor as a single band of approximately 140 kDa using the Western immunoblotting technique carried out by means of a monoclonal anti-Trk antibody. As shown in Fig. 2, anandamide, (*R*)-methanandamide, HU-210, and arvanil, but not palmitoylethanolamide and BML-190, suppress the expression of Trk in starved, β-NGF-treated MCF-7 cells. The effect of anandamide was dose related (IC_{50} = 0.6 μM) and, like that of HU-210, was reversed by SR141716A. The effect of arvanil was partly counteracted by either SR141716A or capsazepine.

HBCCs express CB1 and CB2 mRNA and contain a CB1-immunoreactive protein

As shown in Fig. 3A, the two HBCC lines studied express the CB1 mRNA as detected from the finding of a RT-PCR-amplified cDNA fragment of the expected size obtained by using oligonucleotide primers with the same sequence as human CB1 cDNA (see Ref. 22 for a review). HBCCs also express CB2 mRNA as detected by using the same technique (23). When using the same amount of total RNA (from the same preparation), and the same nonsaturating number of amplification cycles, the CB2 signal was reproducibly much less intense than the CB1 signal, and, in T-47D cells, hardly visible. Using the same procedure, probes and amplification conditions, CB2 and CB1 transcripts of the same size were found in human lymphocytes, the former always in higher amounts than the latter (data

TABLE 2. IC_{50} (micromolar concentration) of different drugs for the inhibition of MCF-7 and T-47D basal cell proliferation

	MCF-7	T-47D
Anandamide	1.40 ± 0.90	1.90 ± 0.20
Arvanil	0.40 ± 0.15	0.35 ± 0.03
2-AG	1.40 ± 0.30	5.00 ± 1.10

Data are the mean ± SD (n = 3), except for anandamide, where data are the mean ± SD of two different sets of data (obtained in different periods; see Ref. 13).

TABLE 1. Molecular target(s) of action of the substances used in this study

Substance	Target(s)	Ref. no.
Anandamide	Endogenous agonist of CB1 > CB2 cannabinoid receptors	2, 5, 6
2-AG	Endogenous agonist of CB1 = CB2 cannabinoid receptors	3–6
HU-210	Synthetic agonist of CB1 = CB2 cannabinoid receptors	1
Arvanil	Synthetic agonist of CB1 ⩾ CB2 cannabinoid receptors Synthetic agonist of vanilloid receptors	17a
(*R*)-Methanandamide	Synthetic, metabolically stable agonist of CB1 > CB2 cannabinoid receptors	1
Palmitoylethanolamide	A weak ligand of CB1 = CB2 cannabinoid receptors (Ki > 10 μM)	1
BML-190	Synthetic agonist of CB2 ⩾ CB1 cannabinoid receptors	20
SR141716A	Synthetic antagonist of CB1 ⩾ CB2 cannabinoid receptors	1
SR144528	Synthetic antagonist of CB2 ⩾ CB1 cannabinoid receptors	1, 21
Capsazepine	Antagonist of vanilloid receptors	17a

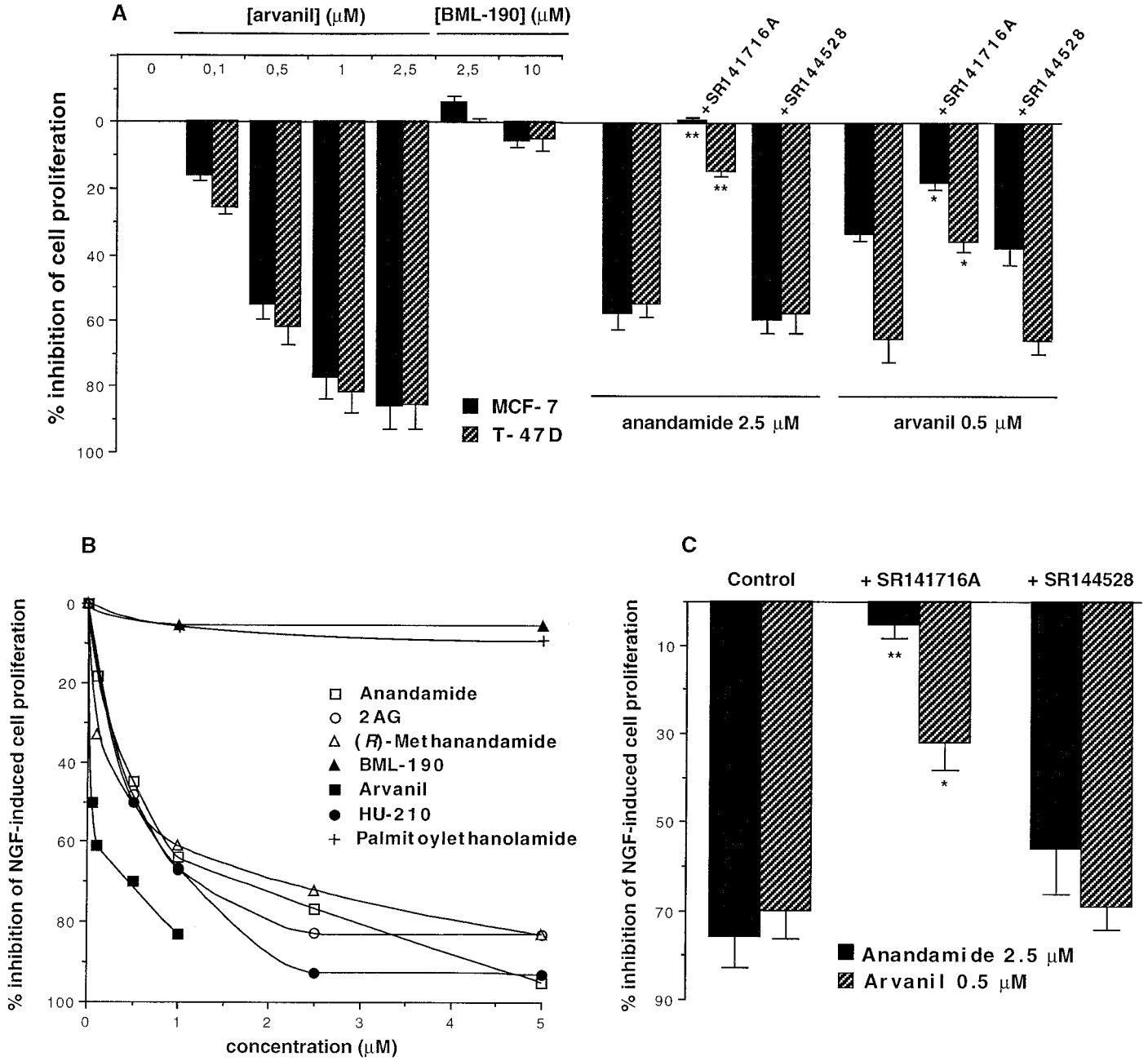

FIG. 1. Effects of endogenous and synthetic cannabimimetics on basal and NGF-induced HBCC proliferation. A, Effects of selective CB1 and CB2 agonists and antagonists on basal HBCC proliferation. The effect is expressed as the percent inhibition of control cell proliferation (average cell proliferation in untreated cells was 50,000–250,000 cells/well·4 days). B, Effects of anandamide, 2-AG, HU-210, metabolically stable anandamide analogs, palmitoyl-ethanolamide, and BML-190 on β-NGF-induced proliferation of MCF-7 cells. The effects are expressed as the percent inhibition of β-NGF-induced proliferation and are the means of 3 (average cell proliferation in cells treated only with NGF was 50,000–100,000 cells/well·2 days). SD bars (never higher than 10–15% of the mean) are not shown for the sake of clarity. C, Effects of CB1 and CB2 selective antagonists on anandamide and arvanil inhibition of β-NGF-induced MCF-7 cell proliferation. A and C, Data are the mean ± SD (n = 3). The concentration of the two antagonists was 0.2 μM, and this corresponds to the maximal inhibitory effect observed at the doses of anandamide and arvanil shown. *, $P < 0.05$; **, $P < 0.01$ (*vs.* anandamide or arvanil).

not shown). The presence of CB1-like receptors in MCF-7 cells was confirmed by Western immunoblotting, which showed the presence of a single band with an apparent molecular mass of approximately 57 kDa (Fig. 3B). This value is compatible with the human CB1 receptor molecular mass if glycosylation of this protein (as described in Ref. 22) is taken into account. This value is very similar to the molecular mass (~58 kDa) of CB1-immunoreactive proteins detected in various rat and human cells that express CB1 receptors (24). We found that the abundance of the CB1 transcript, compared with that of the β_2-microglobulin transcript, as well as the intensity of the CB1-

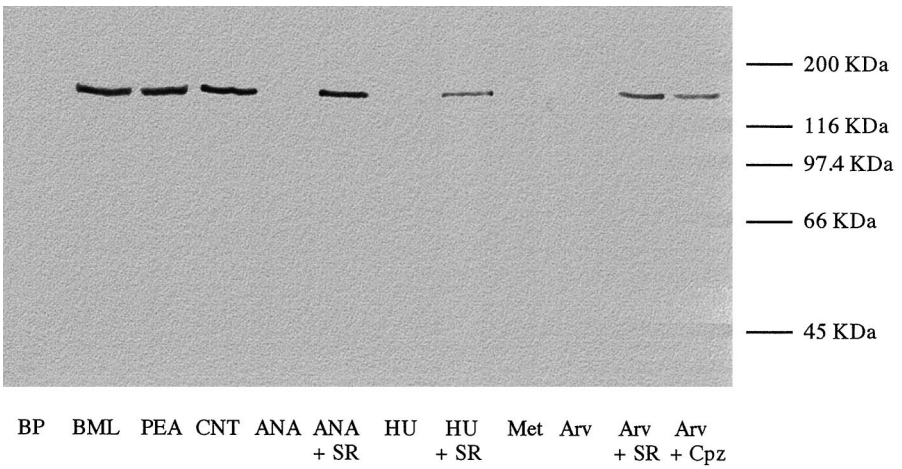

FIG. 2. Endogenous and synthetic cannabimimetics suppress Trk levels in MCF-7 cells as determined by Western immunoblotting. Twenty micrograms of total proteins were loaded on each lane. This blot is representative of three experiments. BML, BML-190 (5 μM); PEA, palmitoylethanolamide (5 μM); CNT, control; ANA, anandamide (1 μM); SR, SR141716A (0.5 μM); Met, (*R*)-methanandamide (1 μM); HU, HU-210 (1 μM); Arv, arvanil (1 μM); Cpz, capsazepine (0.2 μM); BP, blocking peptide.

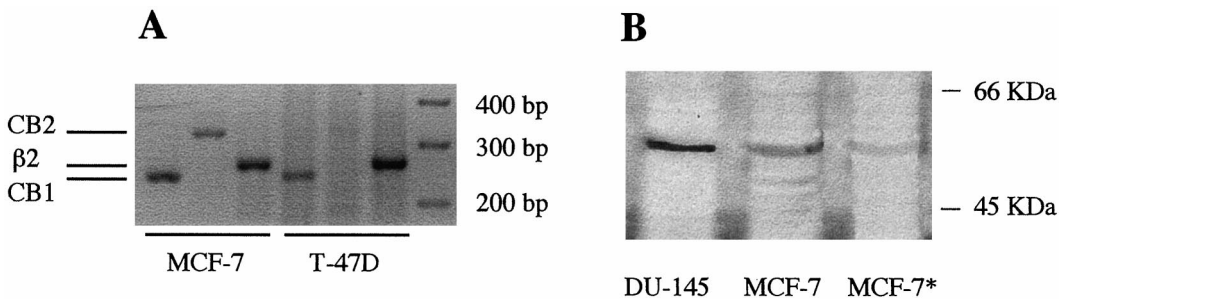

FIG. 3. Molecular identification of CB1-like receptors in HBCCs. A, Identification of CB1 and CB2 transcripts in MCF-7 and T-47D cell RNA by RT-PCR. The mobility of markers and the CB1 and CB2 transcripts of the expected size are shown together with the β_2-microglobulin (β_2) transcript used as the housekeeping gene. B, Western immunoblot of MCF-7 cell proteins carried out with a polyclonal antibody against the CB1 receptor. Results obtained with proteins from DU-145 cells are also presented. Thirty micrograms of total proteins were loaded onto gels in each case. *, Proteins obtained from cells after 12 subculturing passages. Mol wt markers are shown. The gel and the blot are representative of three different experiments.

immunoreactive band decreased when MCF-7 cells underwent 10–15 subculturing passages (Fig. 3b and data not shown).

HBCC membranes contain specific binding sites for [^{3}H]SR141716A

Membrane preparations from MCF-7 and T-47D cells contain specific binding sites for [^{3}H]SR141716A as detected by the filtration binding assays used in this study. Table 3 shows the B_{max} and K_d values for [^{3}H]SR141716A binding sites in each HBCC line, calculated from the corresponding Scatchard plots. [^{3}H]SR141716A specific binding in these two cell lines, determined in the presence of 10 μM SR141716A, depended on the number of subculturing passages and varied between 10–40% for MCF-7 cells and 15–30% for T-47D cells. Anandamide K_i values for the displacement of [^{3}H]SR141716A specific binding were 1.50 ± 0.31 and 0.85 ± 0.25 μM (n = 3) for T-47D and MCF-7 cells, respectively. The K_i value for arvanil in MCF-7 cells was 0.25 ± 0.10 μM (n = 3).

Effects of anandamide and 2-AG on human prostate cancer cell proliferation

In agreement with a previous study (18), DU-145 cells proliferated more rapidly in response to human PRL; a 25 ± 1% increase in cell proliferation (n = 19) was observed with 1 mIU/ml of the hormone after a 3-day treatment. Longer treatments of DU-145 cells with PRL or higher doses of the hormone did not lead to a further increase in cell proliferation (18) (data not shown). Anandamide, 2-AG, and HU-210 were potent inhibitors of PRL-induced, but not basal, DU-145 cell proliferation (Fig. 4A). (*R*)-Methanandamide and arvanil were not less potent than anandamide. The effect of anandamide was reversed by SR141716A, but not by SR144528, and was not mimicked by BML-190 and palmitoylethanolamide (Fig. 4, A and B). Both SR141716A and capsazepine, but not SR144528, counteracted the effect of arvanil (Fig. 4B and data not shown).

DU-145 cells express CB1-like receptors and specific binding sites for [^{3}H]SR141716A

As shown in Fig. 4C, DU-145 cells express CB1 and, to a lesser extent, CB2 mRNA, as determined from the finding of RT-PCR-amplified cDNA fragments. DU-145 cells also contain specific binding sites for [^{3}H]SR141716A, whose B_{max} and K_d values are shown in Table 3, and an approximately 57-kDa protein detected by Western immunoblotting (Fig. 3B). Anandamide displaced [^{3}H]SR141716A from DU-145 cell membranes with a K_i of 160 ± 19 nM (n = 3).

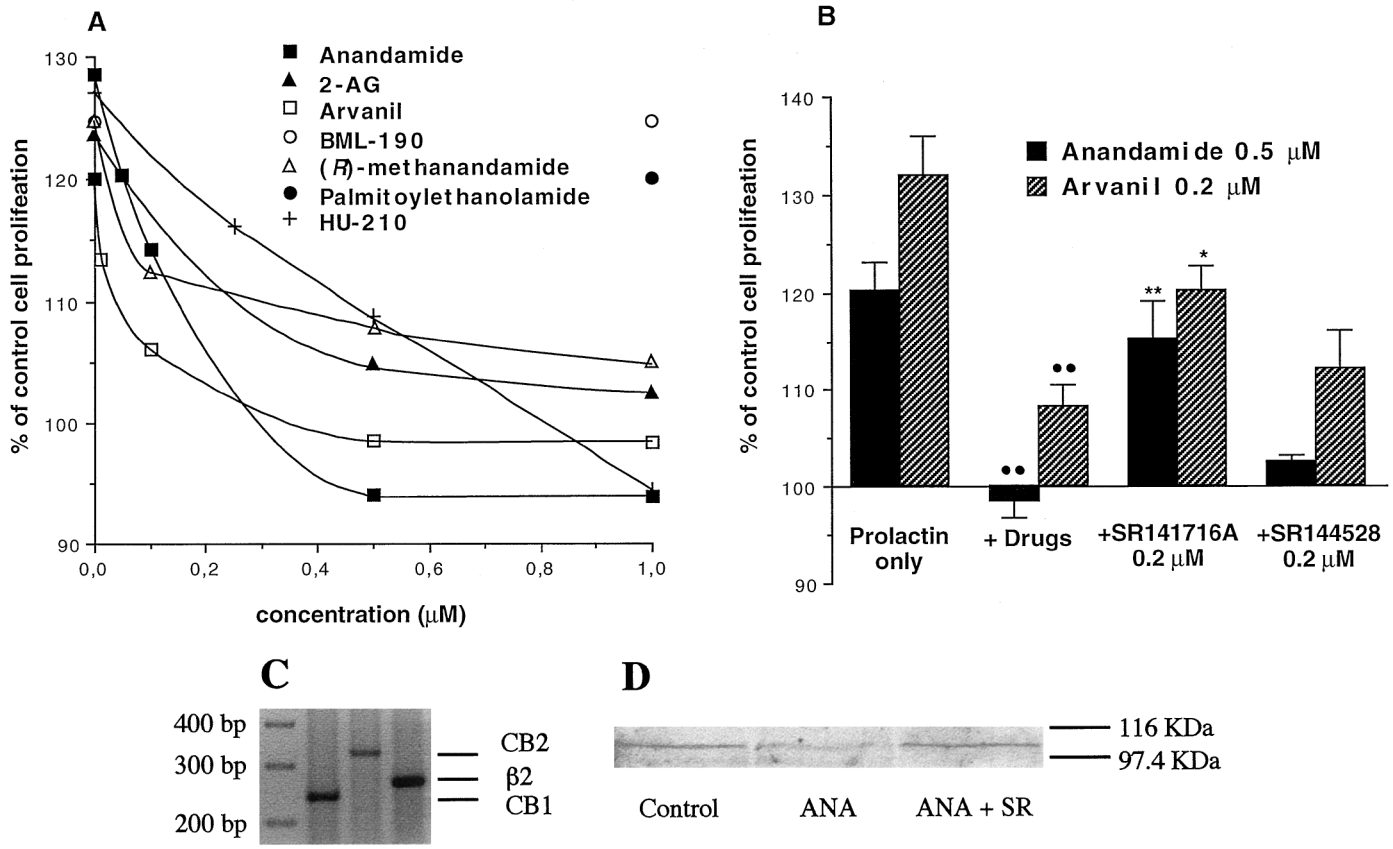

FIG. 4. Antiproliferative effects of ECs on PRL-induced DU-145 cell proliferation. A, Effects of anandamide, 2-AG, metabolically stable anandamide analogs, and CB1 and CB2 agonists on PRL-induced proliferation of DU-145 cells. The effect of exogenous human PRL varied between 120–129% of the vehicle-treated cell proliferation depending on the experiment (average cell proliferation in untreated cells was 50,000–100,000 cells/well·3 days). Anandamide and arvanil exhibited a slight (≤10%) inhibition of basal DU-145 cell proliferation only at high (≥5 μM) doses. The effects of only 1 μM BML-190 and palmitoylethanolamide are shown, but these compounds were also tested at a higher (10 μM) concentration, and no significant effect was found. SD bars (never higher than 10–15% of the mean) are not shown for the sake of clarity. B, Effects of CB1 and CB2 selective antagonists on anandamide and arvanil inhibition of PRL-induced DU-145 cell proliferation. C, Identification of CB1 and CB2 transcripts in DU-145 cell RNA by RT-PCR. For details, see Fig. 3A. D, Western immunoblot of PRLr in DU-147 cells after incubation with vehicle, anandamide (1 μM), or anandamide plus SR141716A (0.2 μM). One hundred micrograms of total proteins were loaded on each lane. Mol wt markers are shown. A and B, Data are the mean ± SD (n = 3). *, $P < 0.05$; **, $P < 0.01$ (*vs.* anandamide or arvanil only). ○, $P < 0.05$; ○○, $P < 0.01$ (*vs.* PRL only). C and D are representative of three distinct experiments.

TABLE 3. Affinity (K_d) and number of sites (B_{max}) for the specific binding of [^{3}H]SR141716A to membrane preparations from HBCCs and prostate cancer cells

	MCF-7	T-47D	DU-145
K_d (nM)	3.80 ± 1.50	0.43 ± 0.19	1.26 ± 0.36
B_{max} (fmol/mg protein)	121.0 ± 35.0	91.1 ± 17.5	94.3 ± 15.0

Data are the mean ± SD (n = 3).

Anandamide inhibits PRLr expression in DU-145 cells

Using the Western immunoblotting technique carried out with a specific monoclonal antibody, DU-145 cells were shown to contain measurable levels of the high molecular mass (~100 kDa) form of the PRLr. Anandamide inhibited the expression of this receptor in a fashion sensitive to the presence of SR141716A (Fig. 4D).

Discussion

We have shown here that 1) ECs inhibit NGF-induced proliferation of HBCCs via suppression of Trk receptors; 2) the inhibition by anandamide of PRLr levels, previously observed in HBCCs, also occurs in another PRL-responsive cell line, the prostate cancer DU-145 cells, and results in the inhibition of PRL-induced cell proliferation; 3) the antiproliferative effects of ECs are mediated by CB1-like receptors. Previously, we reported that the anandamide inhibitory action on basal HBCC proliferation was due to interference with the mitogenic effect of endogenous PRL, which is synthesized by these cells in culture. The sensitivities of different HBCC lines to deprivation of endogenous PRL caused by incubation with a monoclonal antibody against PRL were directly proportional to their sensitivities to anandamide. Furthermore, the effect of the EC was not additive to that of PRL antibody, and anandamide inhibited the action of exogenous human PRL at concentrations lower than those leading to inhibition of basal proliferation. Accordingly, we found that anandamide interferes with PRL action at least in part by down-regulating the expression of the high molecular mass (~100 kDa) form of the PRLr (13). With the present investigation we wanted to determine whether the antimi-

togenic effect of ECs is also observed when cell proliferation is induced by hormones or growth factors other than PRL. This issue is important because cancer cell growth *in vivo* is subject to the control of several of these mediators. An earlier report had shown that synthetic cannabinoids do not interfere with estrogen-induced MCF-7 cell proliferation (25). However, recent studies clearly pointed to NGF as a possible mitogenic agent for HBCCs and prostate cancer cells, both *in vitro* and *in vivo* (19, 26, 27). Therefore, we decided to study the effects of anandamide and 2-AG on NGF-induced HBCC proliferation. We used a well established protocol with MCF-7 cells (19) and observed selectively the effects of drugs on the mitogenic action induced by NGF. We found that submicromolar concentrations of anandamide and 2-AG inhibit NGF-induced MCF-7 cell proliferation in a fashion that was 1) mimicked by metabolically stable cannabimimetic compounds or CB1, but not CB2, receptor-selective agonists; and 2) reversed by CB1, but not CB2, receptor antagonists. Of the two types of NGF-binding sites described to date, the low affinity p75 receptor and the high affinity tyrosine kinase Trk receptors (see Ref. 28 for a recent review), only the latter have been implicated in the tumorigenic activity of NGF. Conversely, coexpression of the p75 receptor with Trk was recently shown to counteract the Trk-mediated proliferative effect of NGF (29). We found here that the inhibition by endogenous and synthetic cannabinoids of NGF-induced MCF-7 cell proliferation was due to down-regulation of Trk receptors. In fact, using the same assay conditions and the same drug concentrations as those necessary to observe the antiproliferative effect, a strong suppression of the levels of the approximately 140-kDa Trk proteins was found with anandamide and other cannabimimetic agents. This effect was reversed by coincubation with a CB1 antagonist. We observed a full correlation between the effects of drugs (or of their combinations) on NGF-induced proliferation and their modulation of Trk levels in MCF-7 cells. This represents the first report of the cannabinoid-induced inhibition of Trk receptors, a family of proteins that is the target of several neurotropins (28).

In a previous study (13) we suggested that anandamide inhibition of basal HBCC proliferation was due to interaction with selective cannabinoid-binding sites and not to the formation of arachidonic acid, a metabolite known to modulate cancer cell proliferation and differentiation (30). The presence of cannabinoid receptors was suggested by binding assays carried out using a radioligand, [^{3}H]CP55,940, that does not discriminate between CB1 and CB2 receptors (1). Therefore, we provided no molecular information as to which cannabinoid receptor subtype was involved in the EC antiproliferative action. In the present study, binding sites for the selective CB1 antagonist [^{3}H]SR141716A were identified, and anandamide shown to bind to these proteins with an affinity comparable to that reported for other CB1-containing tissues and cells (1). Evidence for the presence of CB1 and, to a lesser extent, CB2 mRNA was provided by the RT-PCR technique. Western immunoblotting confirmed the presence of CB1-like receptors in MCF-7 cells. These data taken together with the lack of agonistic or antagonistic effects described above for selective CB2 agonists and antagonists point to the involvement of CB1-like receptors in EC action on both basal [*i.e.* induced by endogenous PRL (13)] and NGF-induced HBCC proliferation. In further support of this conclusion we observed that when MCF-7 cells undergo several subculturing passages they express increasingly lower levels of CB1 mRNA, CB1-immunoreactive protein, and [^{3}H]SR141716A specific binding, and become correspondingly less responsive to anandamide. Furthermore, the K_i values for anandamide displacement of [^{3}H]SR141716A from HBCC membranes correlate well with the IC_{50} values for its inhibition of cell proliferation. Although more potent in EFM-19 cells (13), 2-AG was equipotent to or less potent than anandamide in MCF-7 and T-47D cells, respectively. This fact may be due to different contents of anandamide or 2-AG hydrolases in HBCC lines. Indeed, we showed that 1) such enzymes are present in HBCCs and limit the action of endocannabinoids (13, 17); and 2) ECs are equipotent to or, more frequently, less potent than their metabolically stable or nonhydrolyzable analogs in all HBCC lines studied (this study and data not shown).

A corollary to our previous finding of an involvement of PRLr in anandamide antimitogenic actions on HBCCs is that ECs may inhibit the proliferation of other PRL-responsive cells that express functional cannabinoid receptors. In keeping with this hypothesis, here we have shown that when the proliferation of the prostate DU-145 cell line is induced by exogenous PRL, anandamide and 2-AG are again capable of exerting a potent antiproliferative action, and that anandamide inhibits the expression of PRLr in these cells. Little or no effect was observed on basal DU-145 cell proliferation, which probably depends on other factors present in the cell culture medium. As in the case of HBCCs, this antimitogenic effect was not due to arachidonic acid formed from anandamide or 2-AG hydrolysis, as metabolically stable anandamide analogs were equipotent or more potent than the two ECs. In fact, the effects of anandamide and 2-AG on DU-145 cell proliferation were due to the interaction with CB1 receptors, as we found in these cells specific [^{3}H]SR141716A binding displaceable by anandamide with a K_i consistent with the IC_{50} for its antiproliferative effect. A CB1 mRNA transcript and an abundant CB1-immunoreactive protein were also identified. Moreover, as shown above for HBCCs, selective CB1 and CB2 agonists and antagonists were either very effective or ineffective by either mimicking or counteracting, respectively, the anandamide antiproliferative effect on DU-145 cells. Given the slight (20–30%) stimulatory effect of exogenous PRL on DU-145 cell proliferation *in vitro* observed by us and others (18), further studies are necessary before suggesting that the inhibitory effects of ECs on these cells may have a biological relevance *in vivo*. However, our data with DU-145 cells confirm that both endogenous and synthetic cannabinoids are potent negative effectors of PRL receptor levels in tumor cells that coexpress this protein and CB1 cannabinoid receptors.

In conclusion, our data suggest that ECs may exert a general inhibition of the mitogenic action of PRL and NGF by interfering with the expression of their respective receptors. PRLr and Trk have been shown to be involved in the onset and establishment of breast and prostate carcinomas that seem to be facilitated by endogenous PRL and NGF (18, 19, 26, 27, 31, 32). However, our findings may have important

implications not only for the possible development of new, therapeutically useful, antitumor drugs, but also for understanding of the general physiological role played by ECs in relation to PRL and NGF biological responses. Although PRL is an important mediator in the regulation of reproduction and lactogenesis as well as the immune response (reviewed in Refs. 33 and 34), NGF has been implicated not only in neuronal differentiation (28), but also in neurogenic inflammation, chronic pain, and the neuroendocrine/immune axis (reviewed in Ref. 35). It is possible that down-regulation of PRLr and Trk expression by ECs is also found in other tissues and cells that respond to either of the two growth factors (*e.g.* pituitary lactotroph cells, epithelial mammary duct cells, reproductive organs, lymphocytes, mastocytes, and undifferentiated nerve cells). This would support the hypothesis of a modulatory role of these lipids in situations as diverse as reproduction and lactogenesis, neuronal development, inflammation, antinociception, and the immune response (5–12), which is suggested, *inter alia*, by the occurrence of ECs in the pituitary (36), immune cells (37, 38), and milk (39). It is also possible that the regulatory mechanisms described here are restricted to cancer cells. Therefore, further studies are required to assess in what other cell types and under what conditions the inhibitory effect of ECs on PRLr and Trk levels can be observed. Also, an investigation of the possible mechanism through which activation of CB1 receptors leads to down-regulation of PRLr and Trk should be carried out. It is possible that a typical intracellular event triggered by CB1 receptor activation, adenylyl cyclase inhibition (1), underlies the suppression of Trk, as *trk* gene expression was recently shown to be up-regulated by cAMP in human monocytes, rat brain, and pancreatic β-cells (40–42).

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