The endogenous cannabinoid anandamide inhibits human breast cancer cell proliferation

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ABSTRACT Anandamide was the first brain metabolite shown to act as a ligand of “central” CB1 cannabinoid receptors. Here we report that the endogenous cannabinoid potently and selectively inhibits the proliferation of human breast cancer cells in vitro. Anandamide dose-dependently inhibited the proliferation of MCF-7 and EFM-19 cells with IC50 values between 0.5 and 1.5 μM and 83–92% maximal inhibition at 5–10 μM. The proliferation of several other nonmammary tumoral cell lines was not affected by 10 μM anandamide. The anti-proliferative effect of anandamide was not due to toxicity or to apoptosis of cells but was accompanied by a reduction of cells in the S phase of the cell cycle. A stable analogue of anandamide (R)-methanandamide, another endogenous cannabinoid, 2-arachidonoylglycerol, and the synthetic cannabinoid HU-210 also inhibited EFM-19 cell proliferation, whereas arachidonic acid was much less effective. These cannabimimetic substances displaced the binding of the selective cannabinoid agonist [3H]CP 55,940 to EFM-19 membranes with an order of potency identical to that observed for the inhibition of EFM-19 cell proliferation. Moreover, anandamide cytostatic effect was inhibited by the selective CB1 receptor antagonist SR 141716A. Cell proliferation was arrested by a prolactin mAb and enhanced by exogenous human prolactin, whose mitogenic action was reverted by very low (0.1–0.5 μM) doses of anandamide. Anandamide suppressed the levels of the long form of the prolactin receptor in both EFM-19 and MCF-7 cells, as well as a typical prolactin-induced response, i.e., the expression of the breast cancer cell susceptibility gene brcal. These data suggest that anandamide blocks human breast cancer cell proliferation through CB1-like receptor-mediated inhibition of endogenous prolactin action at the level of prolactin receptor.

Anandamide (N-arachidonoyl-ethanolamine), the first endogenous ligand of central (CB1) cannabinoid receptors, was isolated from porcine brain in 1992 (1). Since its discovery, several CB1-mediated effects have been reported for this endogenous cannabinoid in numerous mammalian tissues (reviewed in refs. 2 and 3). Of special interest for the development of new drugs seem to be the pharmacological actions exerted by anandamide in peripheral tissues. In the cardiovascular system, anandamide induces hypotension and bradycardia (4) and lowers ocular blood pressure (5). In the gastrointestinal and urinary tracts, the cannabimimetic metabolite inhibits smooth muscle contraction (6). Anandamide and CB1 receptors have been suggested to play a modulatory role during uterus–embryo interactions (7). Finally, anandamide and 2-arachidonoyl-glycerol, another putative “endogenous cannabinoid” (8, 9), have been shown to affect lymphocyte and macrophage function (10–12), even though it is not clear yet whether these immunomodulatory actions are mediated by the CB1 or the “peripheral” CB2 cannabinoid receptor subtype.

A neuroendocrine function for anandamide was also proposed on the basis of the interactions between psychoactive cannabinoids and steroid hormone action, described previously and reviewed in ref. 13, and of the finding of anandamide stimulatory or suppressing effects on the serum levels, respectively, of corticosterone or prolactin and growth hormone (14–16). Recently, further insights have been gained on the hypothalamic cellular targets of anandamide that are at the basis of its CB1-mediated regulatory action on the hypothalamo-pituitary-adrenal axis (17, 18).

Based on this background, in the present study we have addressed the question of whether anandamide would exert a modulatory effect on the proliferation of human breast cancer (HBC) cells, which has been suggested to depend on prolactin and estrogens (19–22). Inasmuch as they express prolactin receptors, respond to prolactin treatment, and synthesize their own prolactin (19–22), these cells are similar to B and T lymphocytes, whose proliferation has been shown to be stimulated by the hormone (23, 24) and inhibited by cannabinoids, anandamide (10), and 2-arachidonoylglycerol (11). Therefore, we have investigated the possible anti-mitogenic action of anandamide and other cannabimimetic compounds on two epitheliod HBC cell lines, EFM-19 and MCF-7 cells, that have been used widely in the past for studies on the pharmacology and biochemistry of lactogenic hormones (19–22, 25).

MATERIALS AND METHODS

Cell Proliferation Assays, 3H-Thymidine Incorporation Studies, and Effect on Cell Cycle. Anandamide was synthesized in large amounts and purified as described (1). Arachidonoyl-trifluoromethyl-ketone and (R)-methanandamide were purchased from Biomol (Plymouth Meeting, PA), and arachidonic acid and human prolactin were purchased from Sigma. SR 141716A and HU-210 were gifts from Sanofi Recherche, Montpellier, France, and Prof. Raphael Mechoulam, The Hebrew University of Jerusalem, Israel, respectively. Prolactin mAb was purchased from Pierce. EFM-19, MCF-7, and BT-474 cells, purchased from DSM, Braunschweig, Germany, and T-47D cells, purchased from American Type Culture Collection, were cultured in diaphraged media prepared

Abbreviation: HBC, human breast cancer.

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8375
according to the instructions of the manufacturers except for MCF-7 cells, which were cultured in diaminoethanol modified essential medium containing 5% heat-inactivated fetal bovine serum. These culture media contained no detectable prolactin by radioimmunoassay. Cell proliferation assays were carried out in triplicate by a slight modification of the method described (19) in 6-well dishes containing subconfluent cells (at a density of ~50,000 cells/well). When using EFM-19 and BT-474 cells, which take 24 h to completely adhere to plastic and start growing, substances to be tested were introduced 24 h after cell seeding. With MCF-7 and T-47D cells, which immediately adhere to plastic and start growing, substances to be tested were introduced 6 h after cell seeding. Depending on the experiment, various doses or one single dose of the substances tested were introduced 24 h after cell seeding. Reactions were washed twice with 1 ml of wash buffer every 6 h.

RESULTS AND DISCUSSION

Anandamide dose-dependently inhibited the proliferation of human breast epithelioid EFM-19 cells with an average IC50 value of 1.5 ± 0.3 μM and 92.0 ± 4.0% maximal inhibition at 10 μM (mean ± SEM, n = 7; Fig. 1a). Anandamide was administered daily at each change of the culture medium because cells were found to convert rapidly 1.5 μM [3H]anandamide to [3H]ethanolamine and arachidonic acid with a predicted t1/2 of ~6 h (Fig. 1a Inset). However, when cell proliferation was measured daily, anandamide effect was already noticeable (and maximal) after 48 h of treatment of cells, i.e., when the exponential phase of cell growth is about to start (Fig. 1b). Anandamide anti-proliferative action was due to inhibition of DNA synthesis, measured by determining the incorporation of [3H]thymidine in DNA (see legend to Fig. 1), and was not due to toxic effects or apoptosis of cells, as assessed by testing the effect on cell viability and DNA fragmentation, respectively. Analogous results were obtained with other HBC cell lines, i.e., the widely studied MCF-7 cells, where anandamide effect was even more marked (estimated IC50 = 0.5 μM, 83% maximal inhibition at 5 μM after a 3-day treatment, Fig. 1a) and T-47D or BT-474 cells (estimated IC50 = 1.9 and 6 μM, respectively; data not shown). Conversely, no anti-proliferative effect was observed with a 10-μM concentration of anandamide in several tumoral lines derived from other cell types (e.g., murine neuroblastoma N1E-115 cells, rat leukemia RBL-2H3 basophils, mouse heart endothelioma H5V cells, and mouse J774 macrophages) (data not shown). Of interest, anandamide appeared to inhibit significantly and dose-dependently the GI/G2 transition of the cell mitotic cycle in EFM-19 cells (the decrease of cells in the S phase was 9.9 ± 3.6% and 36.8 ± 9.6% at 1 and 5 μM anandamide, respectively, mean ± SD, n = 3).

Polysaturated fatty acids are known to affect cancer cell proliferation (for a review see ref. 29). Therefore, we performed a series of experiments aimed at assessing whether the effect of anandamide was due to arachidonic acid produced from its enzymatic hydrolysis. We tested arachidonoylthromboxane A2, a factor known to inhibit the cell cycle progression (28), with short modifications. These modifications consisted of the use of 12,000 g pellets from EFM-19 and MCF-7 cells (200 μg/tube), the introduction of phenyl-methyl-sulfanyl-fluoride (Sigma, 100 μM) in the binding buffer, and the use of a higher concentration (300 μM) of radioligand. Nonspecific binding was determined in the presence of 10 μM anandamide or HU-210 and accounted for 51% of total bound radioactivity.

Anandamide was assayed, and cells were trypsinized and counted by a hemocytometer, respectively, after 3 (MCF-7 and T-47D cells) or 6 (EFM-19 and BT-474 cells) days or day by day. This method also allowed us to check cell viability by the addition of trypan blue to aliquots of trypsinized cells. No significant decrease in cell viability was observed with up to 100 μM anandamide. For [3H]thymidine incorporation studies, EFM-19 and MCF-7 cells were synchronised in G0/G1 for 40 h with the 3-hydroxy-3-methylglutaryl-CoA reductase inhibitor lovastatin (10 μM) (26) and treated for 72 h with increasing doses of anandamide before 24-h incubation with 106 cpm [3H]thymidine (5 μCi/mmole, Amersham) plus anandamide. The experiment was terminated after washing the cells twice with ice-cold Hank’s balanced salt solution before addition of ice-cold 10% trichloroacetic acid. Radioactivity and DNA content were measured in the trichloroacetic acid precipitate. The effect of anandamide on cell cycle progression was studied in cells fixed with ethanol and stained with propidium iodide.

DNA content was measured by FACStar flow cytometry as previously described by us (26). Possible apoptotic effects of anandamide in EFM-19 and MCF-7 cells were studied by DNA fragmentation and FACStar flow cytometry (26).

[3H]Anandamide Hydrolysis by Cells. The time-dependent hydrolysis of [3H]anandamide (60,000 cpm, 1.5 μM in 6 ml of serum-free culture medium) by intact, sub-confluent EFM-19 cells (in a 100-mm Petri dish) was measured as [3H]ethanolamine produced per 500 μl of incubation medium as described (27).

Polyunsaturated fatty acids are known to affect cancer cell proliferation (for a review see ref. 29). Therefore, we performed a series of experiments aimed at assessing whether the effect of anandamide was due to arachidonic acid produced from its enzymatic hydrolysis. We tested arachidonoylthromboxane A2, a factor known to inhibit the cell cycle progression (28), with short modifications. These modifications consisted of the use of 12,000 g pellets from EFM-19 and MCF-7 cells (200 μg/tube), the introduction of phenyl-methyl-sulfanyl-fluoride (Sigma, 100 μM) in the binding buffer, and the use of a higher concentration (300 μM) of radioligand. Nonspecific binding was determined in the presence of 10 μM anandamide or HU-210 and accounted for 51% of total bound radioactivity.

Immunoprecipitation and Western Immunoblotting. EFM-19 or MCF-7 cells, treated with either vehicle, anandamide (2.5 μM), or anandamide plus SR 141716A (0.5 μM), were washed twice with 137 mM NaCl, 3 mM KCl, 12 mM Na2HPO4, and 2 mM KH2PO4 (pH 7.4) and then lysed with a lysis buffer consisting of 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 150 mM NaCl, 1 mM Na2VO4, 1 mM NaF, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM phenylmethyl-sulfanyl-fluoride, and 1 μg/ml each aprotinin, leupeptin, and pepstatin A. Triton X-100 (1%) also was added for determination of the bracal protein. Immunoprecipitation of prolactin receptor was carried out with 2 μg of an anti-prolactin receptor mAb (U5, purchased from Affinity Bioreagents, Golden, CO) on 1 mg of total proteins for 1 h at 4°C. A suspension of anti-mouse IgG agarose (20 μl, corresponding to 8 μg of IgG, Sigma) then was added, and the mixture was incubated overnight at 4°C. The pellet was washed five times with 1 ml of lysis buffer, resuspended in 20 μl of electrophoresis sample buffer, and boiled for 5 min before loading onto the SDS/polyacrylamide gel. SDS/PAGE of immunoprecipitated proteins (for prolactin receptor analysis) and total proteins (for bracal protein analysis, 50 μg) were carried out on gels containing, respectively, 10% and 7.5% polyacrylamide. Proteins were transferred to nitrocellulose membranes, which then were incubated first for 1 h at room temperature with the first antibody, i.e., anti-prolactin receptor mAb (1:1000), anti-phosphotyrosine polyclonal antibody (1:1000, Amersham), or anti-bracal protein polyclonal antibody (K-18, 1:100, Santa Cruz Biotechnologies) and then with the appropriate horse-radish peroxidase-labeled second antibody conjugates (1:5000, enhanced chemiluminescence, Amersham).

Data Analysis. Data from cell proliferation experiments were expressed as mean ± SEM (or SD) of percentage of cell proliferation in untreated cells and were compared by using the unpaired Student’s t test (level of significance P < 0.05).
No data on the presence of cannabinoid receptors in HBC cells have been reported to date (33). Therefore, we next wanted to determine whether anandamide anti-mitogenic action was due to interaction with selective binding sites or rather to noncannabinoid receptor-mediated intracellular effects (13, 28). We found that a synthetic cannabinoid, HU-210 (34), as well as another endogenous ligand of cannabinoid receptors, 2-arachidonoyl-glycerol (8, 9), but not anandamide, palmitoylethanolamide [which is inactive at CB1 receptors (28, 34)], also exhibited a potent anti-proliferative effect. Anandamide also displaced [3H]CP-55,940, from MCF-19 cell membranes (data not shown). We have not investigated the reasons why SR 141716A exerted a slight anti-proliferative action or why HU-210, as opposed to what previously has been observed for this compound (34), was less active than anandamide in binding assays with EFM-19 cell membranes (data not shown). We have not investigated the reasons why SR 141716A exerted a slight anti-proliferative action or why HU-210, as opposed to what previously has been observed for this compound (34), was less active than anandamide in binding assays with EFM-19 cell membranes. It is possible that some HBC lines express a CB1-like variant whose interactions with SR 141716A and HU-210 are slightly different from those described for CB1 receptors. It is noteworthy that cannabinoid receptor variants have been observed in human tumoral T cells (37).

We investigated the possible mechanism of action through which anandamide and other cannabinoids inhibit cell prolif-
high amounts (for example 0.35 μM) and arachidonic acid (AA, 1 μM), (c) Displacement of [3H]CP 55,940 from EFM-19 cell membrane preparations by anandamide (AEA), HU-210, 2-arachidonoylglycerol, SR 141716A, and palmitoylethanolamide. In a and b, data are mean ± SD (n = 3) and are expressed as a percentage of control cell proliferation (a), or bound per mg of membranes (b). Data from four to six experiments are shown. Asterisks indicate statistically significant differences from data without SR 141716A. *P < 0.05; **P < 0.01. N.S., not significant.

Fig. 2. A CB1-like cannabinoid receptor mediates anandamide effect on EFM-19 cell proliferation. (a) Dose-related effects of two cannabimimetic compounds, HU-210 and 2-arachidonoylglycerol, and of a non-CB1 agonist, palmitoylethanolamide, on EFM-19 cell proliferation, as compared with anandamide. (b) Effect of two different doses of the CB1 antagonist SR 141716A on the anti-proliferative action of anandamide (1 and 2.5 μM) and arachidonic acid (AA, 1 μM), (c) Displacement of [3H]CP 55,940 from EFM-19 cell membrane preparations by anandamide (AEA), HU-210, 2-arachidonoylglycerol, SR 141716A, and palmitoylethanolamide. In a and b, data are mean ± SD (n = 3) and are expressed as in Fig. 1 a, c, and d. In c, data are expressed as percentages of [3H]CP 55,940 bound to membranes, are means of triplicates, and are representative of three distinct experiments. To avoid confusion, we do not show SD bars. Asterisks indicate statistically significant differences from data without SR 141716A. 

We started from the observation that anandamide effects appeared to be restricted to cancer cell lines known to express estrogen and/or prolactin receptors and to proliferate in response to the treatment with steroid and/or lactogenic hormones (19–22). Both the levels and actions of these hormones have been shown to be influenced by synthetic cannabimimetics and anandamide (13–18). Therefore, we hypothesized that the latter compounds could exert their anti-proliferative effect by interfering with the action of one or more of such hormones. However, in a thorough study carried out in MCF-7 cells (38), no interaction between cannabimimetic compounds and estrogen receptors was observed, even though 1 μM desacetyllevonantradol, a synthetic cannabinoid, was shown to inhibit transcriptional activity when tested under conditions (48-h treatment of cells) similar to those used in the present study to detect anandamide anti-proliferative action in the same cell line. As to prolactin, this hormone is produced in high amounts [for example 0.35 μg/ml/day (20)] by most breast cancer cell lines studied so far as well as by human breast carcinomas and was proposed to act as a major autocrine proliferative agent for these cells (19–22, 25) because of its capability of accelerating the G1/S transition of the cell mitotic cycle (24). Accordingly, in EFM-19 cells, exogenous human prolactin (50 ng/ml) enhanced cell proliferation to an extent (23.7 ± 3.6%, mean ± SD, n = 3) comparable to that (10–60%) observed previously with HBC cell lines, including MCF-7 and T-47D cells, which, because of the synthesis of their own prolactin in higher amounts, are less responsive to the exogenously added hormone (25). Furthermore, an almost total blockade of proliferation was observed previously in MCF-7 cells treated with either a prolactin receptor antagonist (25) or a prolactin mAb (20). Here, we found that daily treatment with a mAb against prolactin interfered with the proliferation also of EFM-19, BT-474, and T-47D cells (Fig. 3a and data not shown). Of interest, those cell lines whose proliferation was most sensitive to anandamide (e.g., MCF-7, EFM-19, and T-47D cells) were also the ones that better responded to prolactin antibody treatment (70–98% inhibition of growth with 20 μg/ml of antibody), whereas in BT-474 cells, for which the lowest effect of anandamide was found (see above), only a 29.1% inhibition of proliferation was observed. Thus, the potency of anandamide appeared to parallel the degree of dependency of HBC cell proliferation on endogenous prolactin. However, in the presence of a submaximal concentration of anandamide, the decreased availability of free endogenous prolactin caused by treatment with submaximal doses of prolactin antibody did not result in a further inhibition of proliferation (Fig. 3a). This result indicates that, if prolactin is the target of anandamide anti-proliferative action, the endogenous cannabinoid is not acting by reducing prolactin levels. On the other hand, low doses (0.1–0.5 μM) of anandamide, which were ineffective in the absence of exogenous prolactin, reverted the proliferation of EFM-19 cells induced by 50 ng/ml of the hormone. This effect was blocked by SR 141716A (Fig. 3b). These data, taken together, may suggest that anandamide anti-proliferative effect is caused, at least in part, by interference with the prolactin receptor-mediated proliferative action of endogenous prolactin. A reduction of prolactin receptors by anandamide, for example, would explain why the decreased availability of endogenous prolactin caused by prolactin antibody did not potentiate anandamide anti-proliferative action (Fig. 3a). This hypothesis was supported strongly by the finding that the brain cannabinoid, under the same conditions leading to inhibition of cell proliferation, exerted a strong down-modulatory effect on the levels of the prolactin receptor. This was detected as a 100-kDa protein in Western immunoblot analyses carried out on EFM-19 cell total proteins immunoprecipitated with an antiprolactin receptor mAb and blotted with the same antibody (Fig. 3c, lanes A and B) or with an anti-phosphotyrosine polyclonal antibody (Fig. 3c, lanes D and E). Similar results were obtained in MCF-7 cells (data not shown). Normal and transformed breast cancer cells have been shown to express the long, 100-kDa form of the prolactin receptor (25), which undergoes tyrosine phosphorylation after its binding to prolactin and subsequent homodimerization (for a review see ref. 39). The inhibitory effect of anandamide on prolactin receptor levels probably is mediated by a CB1-like receptor as it was reversed by co-incubation of cells with SR 141716A (Fig. 3c, lanes C and F).

Finally, having found that anandamide interferes with prolactin action at an up-stream level, we wanted to assess whether the endogenous cannabinoid would also inhibit one of the more downstream effects associated with the proliferative action of prolactin and other hormones, i.e., the expression of the breast cancer susceptibility brca1 gene, a marker for...
proliferating and differentiating human mammary epithelial cells (40). The product of this gene recently was shown to peak immediately before the G1/S transition [which is induced by prolactin (24) and impaired by anandamide (see above)] and was suggested to function as a feedback transcription factor that blocks the cell cycle and is triggered by proliferative stimuli (41, 42). We found that the levels of the 220-kDa protein encoded by the *brca1* gene (detected by means of Western immunoblot analyses carried out on EFM-19 cell total proteins blotted with a polyclonal antibody against the *brca1* protein) peaked between 4 and 5 days from cell seeding (data not shown). Three-day treatment of EFM-19 cells with anandamide significantly down-regulated the levels of the *brca1* protein (Fig. 3d, lanes A and B). Again, this effect was reversed by the CB1 antagonist SR 141716A (Fig. 3d, lane C). Similar results were obtained in MCF-7 cells (data not shown).

In conclusion, we have shown that anandamide is a potent and selective inhibitor of the proliferation of HBC cells and that activation of a cannabinoid receptor, whose occurrence had never been described previously in these cells, is at least in part responsible for this effect. We have also provided evidence that anandamide anti-proliferative action may be due to suppression of prolactin receptor synthesis and, therefore, prolactin action, thus resulting in the down-regulation of the *brca1* gene product. These data may have both physiological and pharmacological implications. By means of double isotope-labeling and MS techniques, we have found that anandamide and other fatty acid ethanolamides, together with their biosynthetic precursors, the N-acyl-phosphatidyl-ethanolamines (27, 43), are synthesized by EFM-19 cells, which also express a fatty acid amide hydrolase-like enzyme capable of rapidly terminating anandamide action (44). Therefore, anandamide may function as a local signal for the control of prolactin mitogenic action in HBC cells. Moreover, if found to occur also in lymphocytes, the inhibitory effect by anandamide on prolactin receptors described here may be correlated to the previously reported (10, 11) anti-proliferative action of endogenous cannabinoids on B and T cells, on which prolactin has been proposed to act as an endogenous proliferative agent (23, 24). Experiments aimed at exploring this possibility, as well as at understanding the mechanism of action through which cannabinoid receptor activation leads to suppression of prolactin receptor levels, are now in progress. Based on the results described here, synthetic compounds can be designed in the future by using anandamide and 2-arachidonoyl-glycerol chemical structures as templates, and novel inhibitors of HBC cell proliferation can be developed.

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Immunoblotting was performed with a monoclonal anti-prolactin receptor antibody (c, lanes A–C), polyclonal anti-phosphotyrosine antibody (c, lanes D–F), or a polyclonal anti-*brca1* protein antibody (d, lanes A–C). Proteins immunoprecipitated with a monoclonal anti-prolactin receptor antibody or total proteins (50 μg) were used in c and d, respectively. Control experiments (not shown) did not exhibit the bands at 220 or 100 kDa and were carried out with: (i) no proteins, (ii) no first antibody, and (iii) using, as the first antibody, various antibodies other than the ones mentioned above. The mobility of molecular weight markers is shown. Data are representative of at least three separate experiments. Similar data were obtained with MCF-7 cells. Photographs were taken from films exposed with the enhanced chemiluminescence methodology.