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Stage-Specific Excitation of Cannabinoid Receptor Exhibits Differential Effects on Mouse Embryonic Development[1]

Jun Wang,[3] Bibhash C. Paria,[4] Sudhansu K. Dey,[4] and D. Randall Armant[2,3]

C.S. Mott Center for Human Growth & Development,[3] Department of Obstetrics & Gynecology and Department of Anatomy & Cell Biology, Wayne State University School of Medicine, Detroit, Michigan 48201-1415
Department of Molecular & Integrative Physiology,[4] Ralph L. Smith Research Center, University of Kansas Medical Center, Kansas City, Kansas 66160-7336

ABSTRACT

Anandamide (*N*-arachidonoylethanolamine), an arachidonic acid derivative, is an endogenous ligand for both the brain-type (CB1-R) and spleen-type (CB2-R) cannabinoid receptors. We have previously demonstrated that preimplantation mouse embryos express mRNA for these receptors and that the periimplantation uterus contains the highest level of anandamide yet discovered in a mammalian tissue. We further demonstrated that 2-cell mouse embryos exposed to low levels of anandamide (7 nM) or other known cannabinoid agonists in culture exhibit markedly compromised embryonic development to blastocysts and that this effect is mediated by CB1-R. In contrast, the present study demonstrates that blastocysts exposed in culture to the same low levels of cannabinoid agonists exhibited accelerated trophoblast differentiation with respect to fibronectin-binding activity and trophoblast outgrowth. Again, these effects resulted from activation of embryonic CB1-R. There was a differential concentration-dependent effect of cannabinoids on the trophoblast, with an observed inhibition of differentiation at higher doses. These results provide evidence for the first time that cannabinoid effects are differentially executed depending on the embryonic stage and cannabinoid levels in the environment. Since uterine anandamide levels are lowest at the sites of implantation and highest at the interimplantation sites, the new findings imply that site-specific levels of anandamide and/or other endogenous ligands in the uterus may regulate implantation spatially by promoting trophoblast differentiation at the sites of blastocyst implantation.

INTRODUCTION

Cells of the uterine endometrium and conceptus undergo a series of well-coordinated transformations during mammalian embryo implantation. Before invading the endometrial stroma, mouse primary trophoblast cells differentiate to an adhesive stage, which is characterized by increased fibronectin-binding activity (FBA), expression of $\alpha_5\beta_1$ integrin on the apical surface, and initiation of trophoblast outgrowth on fibronectin [1, 2]. This phenotypic transformation of the trophoblast cells is influenced by the maternal environment, which provides biologically active factors that regulate embryonic differentiation and uterine receptivity for implantation [3].

Anandamide (*N*-arachidonoylethanolamine) is a derivative of arachidonic acid that is synthesized in the pregnant mouse uterus at concentrations much higher than those found in the central nervous system [4–6]. Anandamide is one of the endogenous ligands for cannabinoid receptors, which are members of the G protein (G_i)-coupled receptor family [7–9]. Activation of cannabinoid receptors influences multiple intracellular signaling pathways by inhibiting adenylate cyclase activity [10, 11] and attenuating Ca^{2+} influx [10, 12]. Anandamide also activates focal adhesion kinase [13] and mitogen-activated protein kinase [14].

Two types of cannabinoid receptors have been identified. The brain-type cannabinoid receptor (CB1-R) is expressed primarily within the nervous system, and the spleen-type receptor (CB2-R) is expressed in many cells of the immune system [7, 8]. These two receptor isotypes exhibit similar ligand affinity, and the activation of both receptors inhibits cAMP accumulation. However, the activation of CB1-R inhibits N- and Q-type Ca^{2+} channels and activates K^+ channels, while that of CB2-R appears to have no effect on cytosolic levels of either Ca^{2+} or K^+ [10, 12]. Both CB1-R and CB2-R mRNAs are expressed in mouse preimplantation embryos [5], indicating that mouse preimplantation development could be influenced by ligand-receptor signaling mediated by endogenous cannabinoids.

Cannabinoids adversely affect fertilizing capacity of sperm and other reproductive activities [15–18]. Exposure of 2-cell mouse embryos to active cannabinoids is inhibitory to blastocyst cavitation, trophoblast proliferation, and hatching from the zona pellucida, and these effects are mediated by embryonic CB1-R [5, 6, 19]. The levels of uterine anandamide are inversely related to uterine receptivity for implantation; down-regulation is associated with uterine receptivity, while up-regulation is correlated with uterine refractoriness to blastocyst implantation. Further, anandamide levels are highest in the interimplantation sites and lowest at the sites of implantation [6]. Thus, the presence of cannabinoid receptors in embryos and anandamide in the uterus suggests that intracellular signaling initiated by cannabinoid-like ligands plays an important role in regulating periimplantation trophoblast development [5, 19]. Indeed, low levels of anandamide may be requisite for blastocyst growth and differentiation during implantation. Another endogenous cannabinoid-like ligand, *sn*-2 arachinonyl glycerol (2-AG), is produced by the neuronal cells [20]. Whether this lipid mediator is produced by the uterus is not yet known.

To examine the effects of cannabinoids on trophoblast differentiation, blastocysts were exposed during culture to the endogenous cannabinoids, anandamide and 2-AG, and a synthetic cannabinoid receptor agonist, Win55212-2. Working specifically through CB1-R, these agonists promoted blastocyst differentiation and trophoblast outgrowth within a narrow concentration range, as opposed to their

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[2]Correspondence: D. Randall Armant, C.S. Mott Center for Human Growth & Development, Department of Obstetrics & Gynecology, Wayne State University School of Medicine, 275 East Hancock, Detroit, MI 48201. FAX: 313 577 8554; e-mail: d.armant@wayne.edu

inhibitory effects on 2-cell embryos. Our results have physiological implications for the anandamide concentration gradients observed between implantation and interimplantation sites [6].

MATERIALS AND METHODS

Production and Culture of Embryos

B6CBAF$_1$/J mice (Jackson Laboratory, Bar Harbor, ME) were housed in the C.S. Mott Center vivarium at Wayne State University according to National Institutes of Health and institutional guidelines on the care and use of laboratory animals. Females were superovulated at 5–8 wk of age by i.p. injection of 5 IU eCG (Sigma Chemical Co., St. Louis, MO), followed 48 h later with 5 IU of hCG (Sigma). After hCG injection, they were mated overnight with males of the same strain. Blastocysts were recovered 90 h post-hCG by flushing uterine horns with M2 medium (Sigma). All embryos were cultured in Ham's F-10 medium (Sigma) supplemented with 4 mg/ml BSA, 100 U/ml penicillin, and 0.1 mg/ml streptomycin (all from Sigma) using a 5% CO_2 incubator at 37°C.

Cannabinoid Treatment

Anandamide, Win55212-2 (Research Biochemicals, Natick, MA), and 2-AG (kindly provided by Dr. P. Schmid, The Hormel Institute, University of Minnesota, Austin, MN) were dissolved in ethanol and diluted at least 10^6-fold in culture medium. Blastocysts were cultured continuously at 10 embryos per drop in 10-μl drops of medium containing the vehicle (10^{-6} dilution of ethanol) or the indicated concentration of cannabinoids. In some experiments, embryos were washed free of cannabinoids or vehicle after 30 min of incubation and then cultured without further addition of the ligands. For reversal experiments, blastocysts were cultured for 1 h with 8 nM SR141716A, a CB1-R antagonist, or SR144528, a CB2-R antagonist (Sanofi Recherche, Montpellier, France), and then in medium containing 8 nM of antagonist, with or without 7 nM cannabinoid ligands.

Fibronectin-Binding Assay

Fluorescent-green, 1.0-μm-diameter polystyrene microspheres (Bang's Laboratories, Carmel, IN) were coated with FN-120 (Life Technologies, Inc., Gaithersburg, MD), a 120-kDa fragment of fibronectin containing the central cell-binding domain [21], and blocked using α_1-acid glycoprotein, as described previously [1]. Blastocysts were cultured in the absence of an adhesive substratum for 48 or 72 h. FBA up-regulation was achieved by incubating blastocysts for 1 h with 50 μg/ml FN-120 in culture medium at 37°C. Blastocysts were then incubated with 1 U/ml each of heparinase I, II, and III (Sigma) at 37°C for 30 min and rinsed through three drops of PBS containing 10 mg/ml BSA (PBS/BSA). The binding assay was conducted by incubating embryos at 4°C for 30 min in a 0.2% suspension of FN-120-coated microspheres prepared in PBS/BSA. Blastocysts were then washed free of unbound beads by transfer through four drops of PBS/BSA and fixed overnight in 2% paraformaldehyde (Polysciences, Warrington, PA) at 4°C. FBA was directly related to the fluorescence intensity of bound microspheres, which was quantified over the abembryonic pole of each blastocyst using computer-based image analysis described previously by Schultz and Armant [1].

TABLE 1. Effects of anandamide and CB1-R antagonist on trophoblast outgrowth rates

Treatment	T_{50} (h)[a]	n
Vehicle	69	32
Anandamide	56*	22
SR141716A	66	47
Anandamide + SR141716A	68	41

[a] Trophoblast outgrowth was observed for 120 h of culture and T_{50} was estimated using Probit analysis.
* $p < 0.05$, based on Probit analysis.

Trophoblast Outgrowth Assay

As previously described [1, 22], outgrowth assays were conducted on plastic Petri dishes precoated overnight at 37°C with 50 μg/ml FN-120 [21]. The plastic surface was then rinsed three times with fresh culture medium. Blastocysts were drawn carefully through a narrow micropipette to mechanically remove their zonae pellucidae; they were then placed in individual microdrops on the FN-120-coated plate for an additional 96 h of culture. Trophoblast outgrowth was observed using a Leitz (Wetzlar, Germany) Fluovert FU inverted microscope and Hoffman modulation contrast optics at ×200 magnification. The number of blastocysts exhibiting outgrowth was recorded at appropriate intervals between 24 and 120 h of culture and expressed as percentage outgrowth or as the time at which 50% of the blastocysts had commenced outgrowth (T_{50}), as previously described [23]. Trophoblast outgrowth area was measured after 96 h of culture using an image analysis system, as previously described [22–24].

Statistical Analysis

All experiments were repeated at least three times. FBA values were obtained using at least 15 blastocysts, and percentage outgrowth values were based on a minimum of 90 blastocysts, or as indicated in Table 1. Differences in the temporal FBA profiles between treatment groups were tested for significance using a factorial ANOVA. This test indicated whether a shift had occurred in the relative amount of FBA at 48 h and 72 h of culture. Probit analysis was used to compute T_{50} values and statistically compare outgrowth rate [23].

RESULTS

Cannabinoids Promoted Trophoblast Differentiation

As previously reported [1, 2], FBA is developmentally regulated during serum-free culture of blastocysts. The normal developmental profile is distinguished by low levels of FBA after 48 h, high levels at 72 h, and low levels again after 96 h of culture [2]. Accordingly, we observed that fluorescent microspheres coated with FN-120 bound poorly to blastocysts cultured for 48 h (Fig. 1A). To investigate the role of cannabinoid-like ligands in regulating trophoblast differentiation, blastocysts were cultured in medium containing 7 nM of the cannabinoid agonists anandamide, 2-AG, or Win55212-2, and the rate of trophoblast differentiation was monitored. When blastocysts were cultured for 48 h in medium supplemented with anandamide, the microsphere probes revealed an abundance of receptors for FN-120 on the mural trophoblast surface (Fig. 1B), reflecting precocious trophoblast differentiation. Quantification of the fluorescence intensity of the bound FN-120-coated microspheres at the abembryonic pole showed that FBA in-

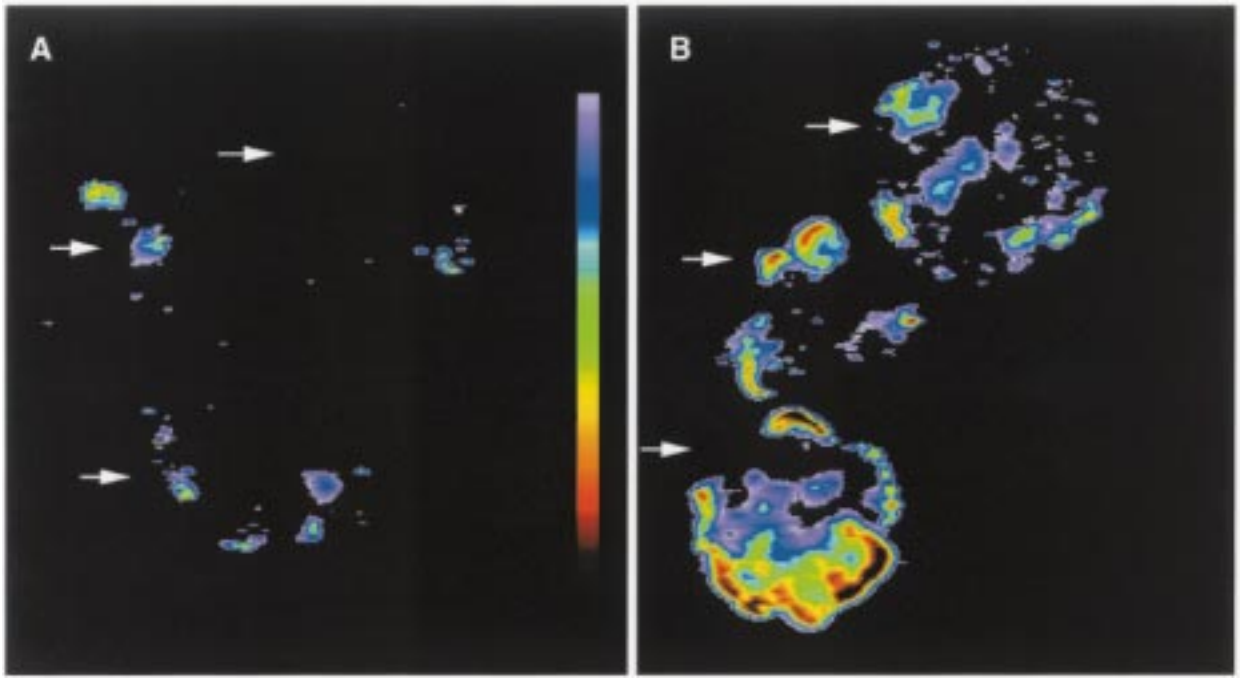

FIG. 1. Accelerated trophoblast differentiation after treatment with cannabinoids. Blastocysts were cultured for 48 h in medium without (**A**) or with (**B**) 7 nM anandamide and then incubated with FN-120-coated microspheres to visualize fibronectin receptors, as detailed in *Materials and Methods.* Fluorescence intensity of the bound microspheres is indicated in pseudocolor according to color bar shown, increasing from top to bottom. Arrows identify the periphery of three blastocysts in each picture.

creased at 72 h in vehicle-treated embryos, while anandamide, 2-AG, and Win55212-2 each produced high FBA after only 48 h of culture (Fig. 2). Cannabinoid-treated embryos exhibited down-regulation of FBA at 72 h, consistent with their accelerated rate of differentiation. Each of the three agonists shifted the developmental profile by approximately 24 h as compared to the vehicle treatment ($p < 0.01$), demonstrating that trophoblast development was accelerated by the activation of embryonic cannabinoid receptors. Blastocysts exposed for only 30 min to cannabinoid agonists exhibited a similar shift in their developmental profile (data not shown), suggesting that even a brief activation of cannabinoid receptors is sufficient to stimulate trophoblast differentiation.

Cannabinoids Functioned through CB1-R but Not through CB2-R

Both CB1-R and CB2-R mRNAs are expressed in preimplantation mouse embryos [5]. Work from other cell systems indicates that anandamide binds to the two receptors with similar affinity and inhibition of cAMP accumulation [10, 12]. To investigate the involvement of these receptors in cannabinoid-induced trophoblast differentiation, we used specific antagonists to CB1-R (SR141716A) or CB2-R (SR144528). SR141716A abrogated the ability of anandamide, 2-AG, or Win55212-2 to accelerate trophoblast differentiation ($p < 0.01$; Fig. 2). During these treatments, FBA did not increase until 72 h; this result was similar to findings in the vehicle-treated embryos. In contrast, SR144528 did not noticeably attenuate cannabinoid-induced acceleration of trophoblast differentiation. This CB2-R-specific antagonist at 10 nM fails to reverse the inhibition of preimplantation development by 7 nM cannabinoids, and a CB2-R-specific agonist was without effect at several-fold above the effective concentration of anandamide [25]. These results establish that the activation of CB1-R, but not CB2-R, stimulates trophoblast cell differentiation.

The treatment of blastocysts with either of the antagonists alone did not influence their differentiation (Fig. 2). However, FBA of blastocysts treated with SR141716A appeared somewhat, albeit not significantly ($p > 0.2$), lower than in vehicle-treated embryos at 72 h, suggesting that the activation of CB1-R through an autocrine mechanism may support trophoblast differentiation.

Cannabinoids Induced Precocious Trophoblast Outgrowth through CB1-R

Trophoblast outgrowth is a reliable marker for trophoblast differentiation and migratory activity on extracellular matrix substrates such as fibronectin and laminin [26]. Therefore, we examined the effects of cannabinoid exposure on trophoblast outgrowth activity on FN-120. Trophoblast cells of blastocysts treated with 7 nM anandamide for 30 min at the outset of culture frequently began to dissociate and form large outgrowths after only 48 h, whereas most vehicle-treated blastocysts remained intact or only exhibited early signs of trophoblast cell adhesion and spreading (Fig. 3, A and B). Anandamide and Win55212-2 treatments shifted ($p < 0.05$, by Probit analysis) the T_{50} for outgrowth from 68 h for vehicle-treated blastocysts to 60 h and 61 h, respectively (Fig. 3C). Similar results were obtained when the cannabinoids remained in the medium throughout the entire culture period (data not shown). Again, the application of CB1-R antagonist blocked the acceleration of trophoblast differentiation induced by cannabinoids (Table 1). These observations, consistent with our FBA experiments, strongly indicated that cannabinoids at a concentration of 7 nM effectively accelerated periimplantation trophoblast differentiation.

To assess the effect of cannabinoid treatment on tropho-

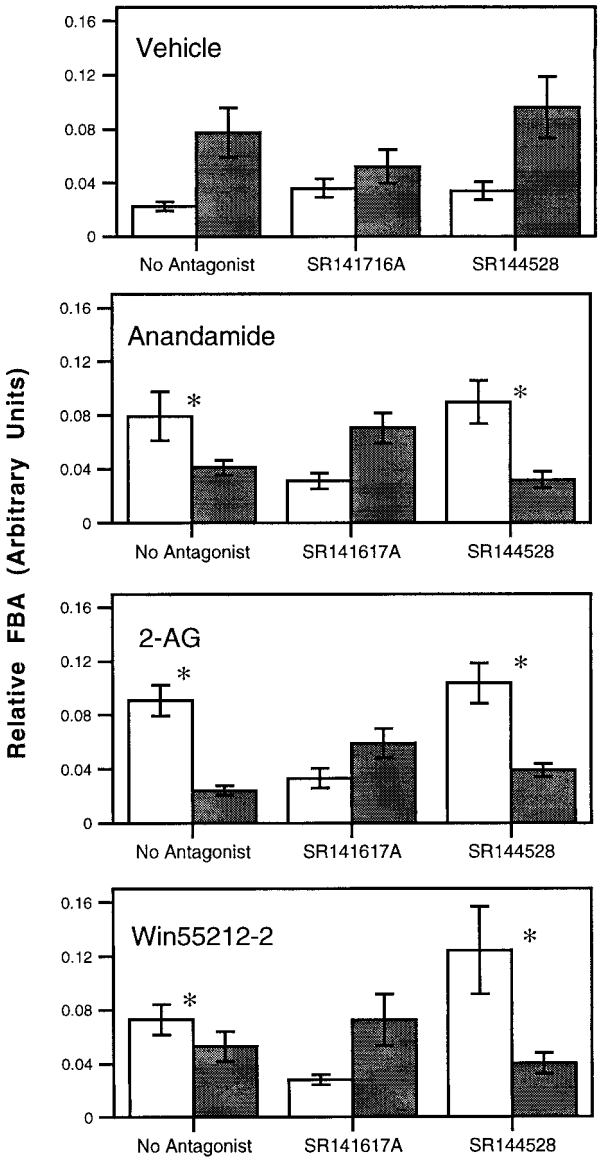

FIG. 2. FBA on the surface of intact embryos was quantified, according to the procedures of Schultz and Armant [1], as the mean ± SEM at 48 h (open bars) or 72 h (solid bars) after initiation of blastocyst culture. Embryos were cultured in medium containing the indicated combinations of 7 nM cannabinoid receptor agonist (anandamide, 2-AG, or Win55212-2) and 8 nM cannabinoid receptor antagonist (SR141716A or SR144528). A factorial ANOVA was used to compare the temporal expression profile of FBA for each treatment to the profile of the control (Vehicle/No Antagonist). Asterisks (*) are placed over the 48-h/72-h pairs where $p < 0.05$.

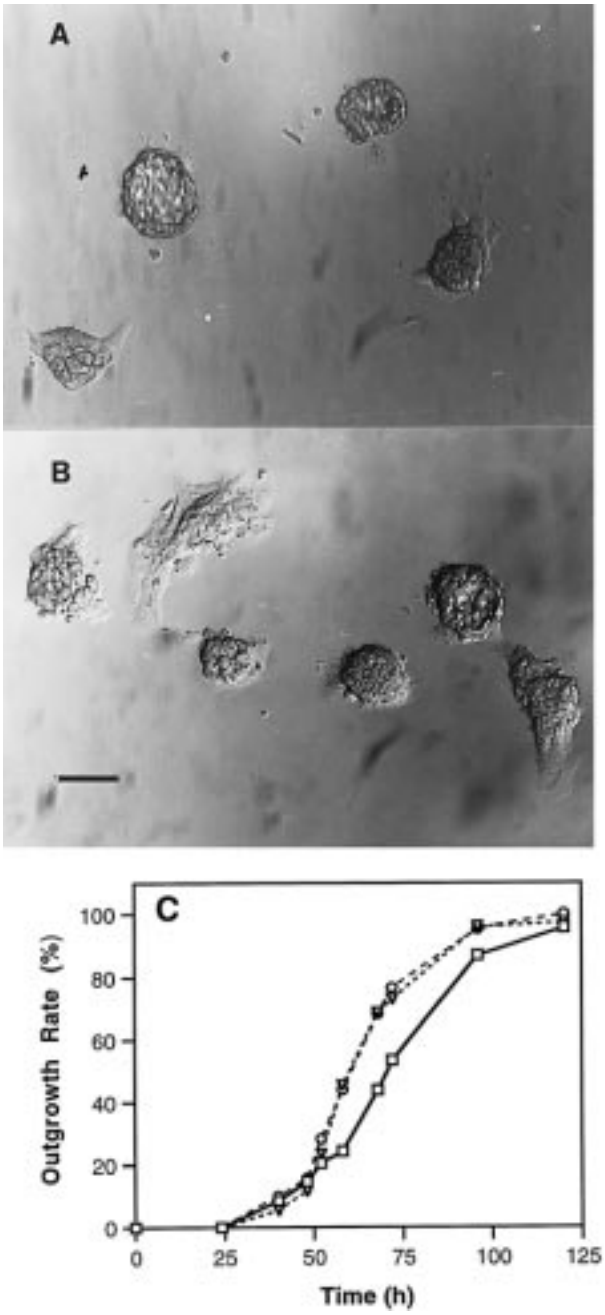

FIG. 3. Trophoblast outgrowth after treatment with cannabinoids. Blastocysts were incubated for 30 min in the absence (**A**) or presence (**B**) of 7 nM anandamide and cultured for an additional 48 h to demonstrate trophoblast outgrowth on FN-120. In **C,** the rate of trophoblast outgrowth was determined after a 30-min treatment with vehicle (squares), 7 nM anandamide (triangles), or 7 nM Win55212-2 (circles). Bar = 100 μm.

blast migratory activity, the area of trophoblast outgrowth was measured. After 96 h of culture, no difference ($p = 0.99$) was observed in outgrowth area between the blastocysts treated with vehicle (35 312 ± 14, 01 μm^2) and anandamide (33 919 ± 11 045 μm^2). Interestingly, although cannabinoids accelerated the rate of trophoblast differentiation, the viability of trophoblast cells may have been compromised. After prolonged outgrowth culture, trophoblast cells are observed that become rounded, nonadherent, or necrotic. Outgrowing trophoblast cells derived from cannabinoid-treated blastocysts showed signs of such degeneration by 96 h, which was not observed for another 24–48 h in vehicle-treated blastocysts. The early loss of outgrowing trophoblast cells was observed whether cannabinoids were present for only 30 min or continuously.

Differential Concentration-Dependent Effects of Cannabinoids on Trophoblast Differentiation

To investigate the concentration-dependent effects of cannabinoids on trophoblast differentiation, blastocysts were cultured in medium containing higher concentrations of anandamide, and FBA was estimated after 48 and 72 h of culture (Fig. 4). Acceleration of trophoblast differentiation was observed when anandamide was present at 7 or 14 nM ($p < 0.05$). However, 28 nM anandamide inhibited trophoblast differentiation, and FBA was not detected at

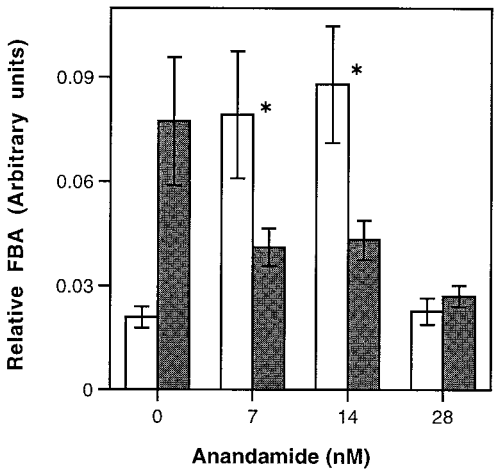

FIG. 4. Differential concentration-dependent effects of anandamide on trophoblast differentiation. Embryos were cultured in medium containing the indicated concentrations of anandamide. FBA was determined after 48 h (open bars) or 72 h (solid bars) of blastocyst culture, as in Figure 2. A factorial ANOVA was used to identify treatments with a temporal expression profile that was different (*, $p < 0.05$) from that for the embryos receiving 0 nM anandamide.

either 48 h or 72 h. These results indicate that cannabinoids at higher concentrations are detrimental to trophoblast development and thus implantation.

DISCUSSION

The ''window'' for successful implantation is defined as the limited time span during which the activated stage of the blastocyst is superimposed on the receptive state of the uterus [3]. The molecular signaling that determines this window of implantation is not yet clearly defined. Because the process of implantation involves coordinated interactions between the embryonic and uterine cells, it could be assumed that ligand-receptor signaling with cannabinoids between the uterus and embryo is involved in this process. This assumption is consistent with our previous observation of expression of cannabinoid receptors in the embryo and synthesis of the endogenous cannabinoid ligand anandamide in uterus. The levels of anandamide in the mouse uterus are inversely correlated with uterine receptivity for blastocyst implantation. Thus, the levels are markedly higher on Day 5 of pseudopregnancy (nonreceptive phase) than on Day 4 (the day of implantation), and the levels at implantation sites are markedly lower than those at the interimplantation sites [6]. We have suggested that higher levels of anandamide are detrimental to blastocyst development and implantation [6]. However, the physiological significance of the presence of anandamide in the receptive uterus and the implantation sites, albeit at lower levels, was not clear. The present investigation suggests that whereas higher levels of cannabinoids adversely affect trophoblast activity and implantation, lower levels are conducive to these events. This is consistent with our recent observation that while single injections of cannabinoids during the preimplantation period fail to affect implantation, sustained delivery of cannabinoids by infusion via miniosmotic pumps interferes with blastocyst functions and implantation [6].

The differentiation-promoting effects of cannabinoids on trophoblast cells at low nanomolar levels are surprising, since similar concentrations were inhibitory at the earlier stages of embryonic development. This stage-specific effect may explain why the preimplantation uterine environment is normally hostile to premorula-stage embryos and why blastocysts fail to survive in the nonreceptive uterine environment. Thus, endogenous cannabinoid-like ligands in the uterus, such as anandamide, may regulate the window of implantation by synchronizing trophoblast differentiation with preparation of the uterus for the receptive state. High concentrations of anandamide in the interimplantation sites may limit trophoblast migrations beyond the implantation sites, as evidenced by the failure of anandamide to stimulate cell migration and the decreased longevity of trophoblast cells exposed to cannabinoids. Therefore, anandamide appears to possess dual functions that may serve to both promote trophoblast invasion at implantation sites and limit invasion beyond those sites.

Cyclooxygenase (COX) is the rate-limiting enzyme in the synthesis of prostaglandins (PGs). COX exists in two isoforms, the constitutive COX-1 and inducible COX-2. We have recently demonstrated that COX-2 is expressed in the uterus solely at the sites of blastocyst apposition during implantation and that targeted disruption of the COX-2 gene, but not COX-1, interferes with implantation in mouse [27]. Since COX-2, but not COX-1, is capable of converting anandamide to PGE_2 ethanolamide [28], it is possible that the relatively lower levels of anandamide at the implantation sites compared with higher levels at the interimplantation sites are regulated by COX-2. COX-2 is able to compete with other enzymes for arachidonic acid, the precursor for synthesis of both PGs and anandamide, suggesting that COX-2 may be involved in regulating the tone of these two lipid mediators conducive to implantation. However, it should be noted that the activity of amidase, an anandamide-hydrolyzing enzyme, is also higher at the implantation sites as opposed to interimplantation sites [29].

The mechanism by which low levels of cannabinoids promote trophoblast differentiation is not yet clearly understood. A key attribute of cannabinoids is their impact on multiple intracellular signaling pathways. We have observed that mobilization of the second messenger, Ca^{2+}, in morulae and blastocysts accelerates cavitation [30] and trophoblast differentiation [23, 30, 31]. Induction of Ca^{2+} signaling at the blastocyst stage alters the expression of over 3% of mRNAs within 1 h [32], which likely modifies the course of subsequent development. The ability of intracellular signaling through Ca^{2+} to stimulate preimplantation development is dependent upon activation of calmodulin [33], a Ca^{2+}-dependent regulatory protein that influences multiple signaling pathways [34]. Reduced long-term survival of trophoblast cells and poor preimplantation development in the presence of low levels of cannabinoids may, therefore, be related to the inhibition of Ca^{2+} influx associated with cannabinoid receptor activation [10, 12]. In blastocysts, low cannabinoid concentrations may activate downstream pathways common to cannabinoid receptors and calmodulin without sufficient disruption of Ca^{2+} homeostasis to preclude the acceleration of trophoblast differentiation. For example, cannabinoids stimulate mitogen-activated protein kinase [14], which is involved in cell proliferation and differentiation; mitogen-activated protein kinase and calmodulin-dependent protein kinases may share downstream substrates that regulate trophoblast development. In summary, this investigation reveals an exciting observation of concentration- and stage-specific functions of endogenous cannabinoid-like molecules during preimplantation embryonic development.

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