Differential G protein-coupled cannabinoid receptor signaling by anandamide directs blastocyst activation for implantation

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Mammalian fertility absolutely depends on synchronized development of the blastocyst to the stage when it is competent to implant, and the uterus to the stage when it is receptive to implantation. However, the molecular basis for the reciprocal interaction between the embryo and the uterus remains largely unexplored. One potentially important mechanism involves signaling between an evolutionarily conserved G protein-coupled protein cannabinoid receptor, CB1, that is expressed at high levels on the surface of the trophectoderm and anandamide (*N*-arachidonoylethanolamine), an endocannabinoid ligand found to be produced at higher levels by the uterus before implantation and then down-regulated at the time of implantation. Using genetic, pharmacological, and physiological approaches, we show here that anandamide within a very narrow range regulates blastocyst function and implantation by differentially modulating mitogen-activated protein kinase signaling and Ca^{2+} channel activity via CB1 receptors. Anandamide at a low concentration (7 nM) induces extracellular regulated kinase phosphorylation and nuclear translocation in trophectoderm cells without influencing Ca^{2+} channels, and renders the blastocyst competent for implantation in the receptive uterus. In contrast, anandamide at a higher concentration (28 nM) inhibits Ca^{2+} channel activity and blastocyst competency for implantation without influencing mitogen-activated protein kinase signaling. Besides uncovering a potentially important regulatory mechanism for synchronizing blastocyst and uterine competency to implantation, this observation has high clinical relevance, because elevated levels of anandamide induce spontaneous pregnancy loss in women.

Natural and endogenous cannabinoids function as ligands for G protein ($G_{i/o}$, GTP-binding proteins sensitive to pertussis toxin)-coupled cannabinoid receptors, CB1 (brain type) and CB2 (spleen type) (1–3). Activation of CB receptors inhibits adenylyl cyclase activity and voltage-gated Ca^{2+} channels but stimulates mitogen-activated protein kinase (MAPK) signaling (4, 5). Cannabinoids exert a broad array of central and peripheral effects including adverse effects on fertilization, embryonic development, and pregnancy (6, 7). Our previous observations of functional expression of CB1 in the preimplantation mouse embryo (8, 9), anandamide synthesis in the uterus (10, 11), and the dose- and stage-specific effects of anandamide on embryo development and implantation suggest that ligand-receptor signaling with endocannabinoids is important for embryo development and implantation (12, 13). The levels of anandamide in the uterus and CB1 in the blastocyst were found to be higher than in the brain during early pregnancy in mice (9, 10). Furthermore, uterine levels of anandamide and blastocyst CB1 were coordinately down-regulated with the attainment of uterine receptivity and blastocyst activation before implantation in contrast to higher levels retained in delayed implanting uterus and dormant blastocysts (10, 11). These results suggested that although the lower levels of anandamide and cannabinoid receptors are beneficial for implantation, the higher levels are detrimental to this process. Indeed, we previously observed that a lower concentration of anandamide stimulates blastocyst outgrowth in culture, but at a higher concentration it inhibits this event (12). Similar biphasic effects of anandamide have also been noted for other physiological functions in mice (6, 14, 15). However, the mechanism of differential effects of endocannabinoids is still unknown. The reduced Mendelian frequency of homozygous mutant offspring born from heterozygous crossing of *CB1* mutant mice (16) and subfertility among CB1 mutant females with 40% pregnancy losses (11) further indicate that regulated endocannabinoid signaling is critical to the establishment of normal pregnancy, although the mechanism by which this signaling influences pregnancy remains mostly unknown. Here, we have combined genetic, pharmacological, cell biological, and physiological approaches to address this question with respect to blastocyst activation and implantation in mice. We show that anandamide at a low concentration activates extracellular regulated kinase (ERK) signaling in dormant blastocysts via their trophectoderm cell surface CB1 and renders them competent for implantation in the receptive uterus. Conversely, anandamide at a higher concentration inhibits Ca^{2+} mobilization and fails to induce blastocyst competency without influencing ERK signaling.

Materials and Methods

Animal Models, Blastocyst Culture/Transfer, and Treatments. Adult WT, *CB1*, and *CB2* mutant mice were housed in the Institutional Animal Care Facility according to National Institutes of Health and institutional guidelines for laboratory animals. The conditions of delayed implantation were induced by ovariectomizing pregnant or pseudopregnant (day 1 = vaginal plug) mice on the morning of day 4 (0830–0900 hours) and maintained by daily injections of progesterone (P_4, 2 mg per mouse) from day 5 until the mice were killed. Activation of dormant blastocysts in P_4-primed delayed implanting pregnant mice was induced by giving a single injection of estradiol-17β (E_2, 25 ng per mouse). To study the effects of anandamide on MAPK signaling, blastocysts were cultured in Whitten's medium in the absence or presence of anandamide (Cayman Chemical, Ann Arbor, MI) as initially described (17). To examine the specificity of anandamide induced activation of MAPK via CB receptors, blastocysts were preincubated with SR141716A or SR144528 (Sanofi Recherche, Montepellier, France) as a CB1- or CB2-selective antagonist (13), respectively, for 1 h before the addition of anandamide. In blastocyst transfer experiments, P_4-primed ovariectomized pseudopregnant WT recipients received an injection of E_2 (3 ng per mouse) to induce receptive uterus (18). Dormant blastocysts exposed to the vehicle or anandamide were

Abbreviations: CB, cannabinoid receptor; E_2, estradiol-17β; ERK, extracellular regulated kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; P_4, progesterone; TS, trophoblast stem.

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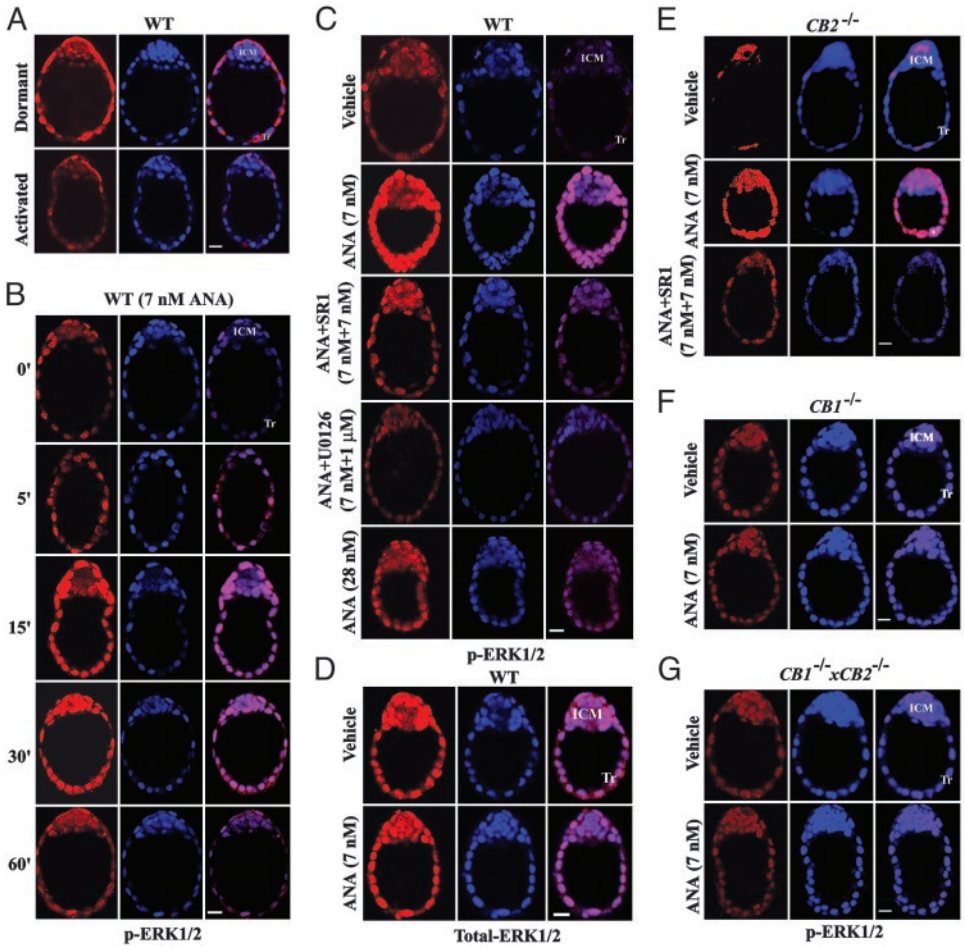

Fig. 1. Activation of ERK by anandamide in dormant blastocysts via CB1. (*A*) Localization of CB1 in dormant and activated blastocysts. The trophectoderm cell surface is decorated with CB1. The levels of CB1 are significantly lower in activated blastocysts than those in dormancy. (*B*) Rapid activation of ERK by anandamide (ANA) in blastocysts. Dormant blastocysts were cultured *in vitro* in the presence of 7 nM anandamide for the indicated times in minutes. Increased phosphorylation of ERK1/2 (*p*-ERK1/2) and its translocation into nuclei were observed in dormant blastocyst trophectoderm cells within 5 min of their exposure to 7 nM anandamide, reaching a peak between 15 and 30 min. (*C*) Activation of ERK by anandamide is dose-dependent. Dormant blastocysts were cultured *in vitro* in the presence of 7 or 28 nM anandamide for 15 min. Anandamide at 7 nM activated ERK1/2 in dormant blastocyst trophectoderm cells, whereas it failed to do so at 28 nM. A CB1-selective antagonist SR141716A (SR1) at 7 nM or a MEK1/2 inhibitor U0126 at 1 μM inhibited the activation of ERK1/2 by 7 nM anandamide. (*D*) Total ERK remained unchanged. No changes in immunointensity for total ERK1/2 were observed in dormant blastocysts exposed to 7 nM anandamide or the vehicle. (*E–G*) Differential activation of ERK signaling by anandamide in *CB* mutant dormant blastocysts. $CB1^{-/-}$, $CB2^{-/-}$, or $CB1^{-/-} \times CB2^{-/-}$ dormant blastocysts were cultured *in vitro* in the presence of 7 nM anandamide. Activation of ERK1/2 in the presence of 7 nM anandamide for 15 min was abrogated by the CB1 antagonist SR141716A (SR1) in $CB2^{-/-}$ blastocyst, but not in $CB1^{-/-}$ or $CB1^{-/-} \times CB2^{-/-}$ blastocysts. Images shown depict TRITC-labeled antigens in red, Hoechst-labeled nuclei in blue, and the merge in pink. (Scale bars, 20 μm.)

then transferred to these recipients 4 h after an E_2 injection. All recipients were killed 24 h after blastocyst transfers, and the number of implantation sites as demarcated by distinct blue bands was recorded after i.v. injections of Chicago blue B dye solution in saline (19). Uteri that were devoid of any sign of implantation were flushed to recover unimplanted blastocysts.

Immunofluorescence Detection of CB1, Ca^{2+} Channel α Subunits, and ERK in Blastocysts. To localize CB1 protein and Ca^{2+} channel α subunits, α_{1A} (P/Q-type), α_{1B} (N-type), and α_{1C} (L-type), as well as to monitor activation of ERK signaling in blastocysts, immunofluorescences were captured in a Zeiss LSM 510 confocal scanning laser microscope as described (20). Rabbit polyclonal antibodies specific to CB1 (1 μg/ml, custom made), or α_{1A}, α_{1B}, and $_{\alpha 1C}$ (1 μg/ml, Alomone Labs, Jerusalem), or total and phospho-ERK 1 and 2 (ERK1/2) (0.5 μg/ml, New England Biolabs) were used.

Ca^{2+} Mobilization and Imaging. Blastocysts were incubated at 37°C for 30 min in Whitten's medium containing 1 μM of the cell-permeant acetoxymethyl ester form of fluo-4 and 0.02% pluronic F127 (Molecular Probes) and 2.5 mM probenecid. Preloaded embryos were washed and transferred into Tyrode's solution (150 mM NaCl/6 mM KCl/1.5 mM $CaCl_2$/1 mM $MgCl_2$/10 mM glucose/10 mM Hepes, pH 7.4) containing 2.5 mM probenecid, then incubated in the presence or absence of different concentrations of anandamide and/or selective antagonists to CB1 or CB2 for 30 min. The stimulus for voltage-gated Ca^{2+} channel activation in blastocysts was a rapid increase in KCl concentration from 6 to 60 mM in the bath solution (21). To chelate extracellular Ca^{2+}, 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetate (BAPTA, 1 mM) was used in the bath solution instead of $CaCl_2$ (22). To identify the relative importance of different Ca^{2+} channel subtypes in blastocyst function, dormant blastocysts were exposed to 2 μM of ω-Conotoxin GVIA and Calciseptine, selective blockers of N- and L-type Ca^{2+} channels (22), respectively, or to 4 μM (+/−)-Bay K 8644, a selective activator of the L-type Ca^{2+} channel (23), in the presence or absence of KCl stimulation. Images were acquired

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for 5 min after the addition of KCl on a Zeiss LSM 510 confocal scanning laser microscope.

Culture of Trophoblast Stem Cells and Treatment with Cannabinoid Agonist. A trophoblast stem (TS) cell line derived from mouse blastocysts was used (24). Immunolocalization of cannabinoid receptors in this cell line was performed as described above and in ref. 20. TS cell culture and an embryonic fibroblast conditioned media (EMFI-CM) were prepared as described (24). The TS cell line was maintained in a proliferative state in a medium containing 70% EMFI-CM, 30% TS cell medium, FGF4 (25 ng/ml), and heparin (1 μg/ml, Sigma) (24). To study cannabinoid signaling via MAPK activation, TS cells were precultured in the TS medium free of serum, FGF-4, and heparin (differentiation conditions) for 5 h and then challenged with CP55,940, a synthetic cannabinoid agonist (Pfizer Diagnostics). After termination of culture, TS cells were lysed, and lysates (50 μg per sample) were analyzed by immunoblotting for total or phosphor-ERK1/2 (25). Targeted protein bands were visualized by using chemiluminescent detection reagents (Pierce) and quantitated by using a Molecular Dynamics personal densitometer.

Results and Discussion

CB1 Is Up-Regulated in Dormant Blastocysts. We have previously shown that the levels of anandamide and CB1 are coordinately down-regulated in receptive uteri and activated blastocysts, respectively, before implantation compared with higher levels in delayed uteri and dormant blastocysts that are incompetent for implantation (11). Our present finding of dramatically up-regulated CB1 protein in the trophectoderm of dormant blastocysts and its rapid down-regulation with the termination of delayed implantation with blastocyst activation by estrogen (Fig. 1*A*) further supports the hypothesis that although lower levels of anandamide and cannabinoid receptors are beneficial for implantation, higher levels are unfavorable to this process.

Anandamide Rapidly Activates ERK Signaling in Blastocysts in a Dose-Dependent Manner via CB1. We sought to address the mechanism of differential effects of anandamide in blastocyst function and implantation. We first examined whether anandamide at varying levels differentially influences MAPK signaling in blastocysts undergoing experimentally induced dormancy (18, 26, 27). We observed that anandamide at 7 nM rapidly induced phosphorylation of ERK and its translocation into nuclei of trophectoderm cells of dormant blastocysts within 5 min, showing a peak between 15 and 30 min (Fig. 1*B*). In contrast, anandamide at 28 nM failed to induce activation of ERK under similar conditions (Fig. 1*C*). In addition, a CB1-selective antagonist SR141716A or a MAPK/ERK kinase (MEK1/2) inhibitor U0126 attenuated ERK phosphorylation in the presence of 7 nM anandamide (Fig. 1*C*). Pertussis toxin, an inhibitor of $G_{i/o}$ protein-coupled receptors, also attenuated ERK phosphorylation in the presence of anandamide (data not shown). However, total ERK content remained unchanged in dormant blastocysts exposed to 7 nM anandamide or the vehicle (Fig. 1*D*). These results provide evidence that ERK activation by anandamide is mediated by $G_{i/o}$ protein-coupled CB1 and suggest that the dose-dependent biphasic function of anandamide in activating ERK signaling via CB1 is physiologically relevant and important for regulating embryonic function for implantation.

To provide genetic evidence that anandamide induced ERK activation in blastocysts is mediated by CB1, we used $CB1^{-/-}$, $CB2^{-/-}$ and $CB1^{-/-} \times CB2^{-/-}$ double mutant blastocysts. Similar to WT blastocysts, anandamide at 7 nM activated ERK phosphorylation in $CB2^{-/-}$ blastocysts (Fig. 1*E*) but failed to induce ERK phosphorylation in $CB1^{-/-}$ or $CB1^{-/-} \times CB2^{-/-}$ dormant blastocysts (Fig. 1 *F* and *G*). In addition, the selective CB1 antagonist SR141716A inhibited ERK activation by anandamide in $CB2^{-/-}$ dormant blastocysts (Fig. 1*E*). ERK activation was also noted in day 4 WT blastocysts by anandamide at 7 nM but not at 28 nM (Fig. 2). Collectively, these results provide genetic and pharmacological evidence that CB1 is the functional cannabinoid receptor, mediating anandamide singling for MAPK activation in the blastocyst. This is consistent with our previous observation of resistance of $CB1^{-/-}$ or $CB1^{-/-} \times CB2^{-/-}$ embryos, but not WT or $CB2^{-/-}$ embryos, to anandamide-mediated effects on preimplantation development *in vitro* (11) and that a CB2-selective antagonist fails to displace anandamide binding to blastocysts (data not shown).

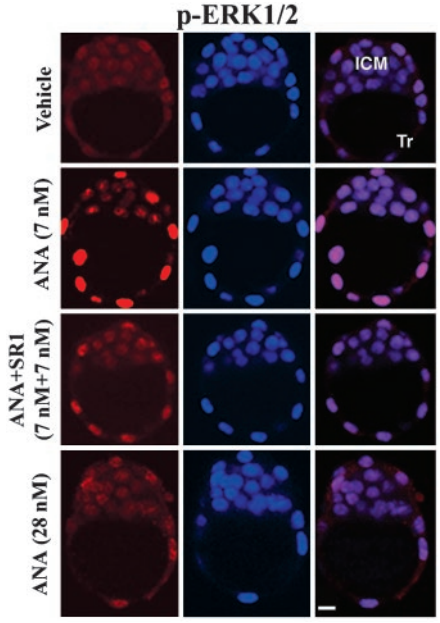

Fig. 2. Activation of ERK by anandamide in normal day-4 blastocysts via CB1. Day-4 blastocysts were cultured *in vitro* in the presence of 7 or 28 nM anandamide (ANA) for 15 min. Activation of ERK1/2 at lower (7 nM) but not higher (28 nM) anandamide concentration was observed. A CB1-selective antagonist SR141716A (SR1) inhibited the accumulation of phospho-ERK1/2 (*p*-ERK1/2) by 7 nM anandamide. Images depict TRITC-labeled antigen in red, Hoechst-labeled nuclei in blue, and the merge in pink. (Scale bar, 20 μm.) Tr, trophectoderm; ICM, inner cell mass.

Cannabinoid Agonist Rapidly Activates ERK Signaling via CB1 in Differentiating TS Cells. Limited availability of blastocyst tissues prompted us to use a mouse TS cell line, originally generated by Janet Rossant's group (24), to obtain more quantitative information with respect to ERK signaling in response to cannabinoids. We observed that the differentiating TS cells are decorated with CB1 on their surfaces (Fig. 3*A*), similar to the expression pattern on the blastocyst trophectoderm cell surface (Fig. 1*A*). We then asked whether CP55,940, a synthetic cannabinoid agonist, could induce ERK phosphorylation in TS cells; this agonist is effective in inhibiting implantation via CB1 when maintained at an elevated level (10). Based on results in blastocysts, we initially examined the dose-dependent effects of CP55,940 on ERK activation in TS cells over a period of 15 min. We observed that CP55,940 at 7 nM significantly induced ERK1 phosphorylation with a peak activation at 28 nM, but increasing the concentrations (112 and 448 nM) of CP55,940 did not further enhance ERK1 phosphorylation; rather, a decreasing trend was noted. We then examined the time-dependent activation of ERK phosphorylation at 7 nM of CP55,940 and found that maximal activation occurs at 5 min (Fig. 3 *B* and *C*). A MEK1/2 inhibitor U0126 or the selective CB1 antagonist SR141716A, but not the

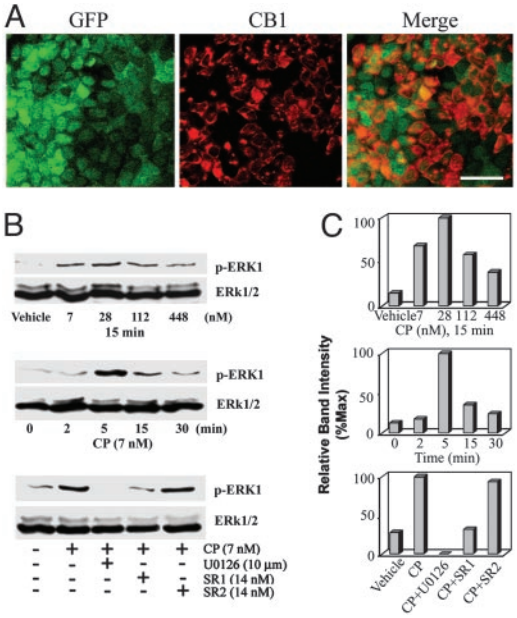

Fig. 3. Cannabinoid agonist CP55,940 induces activation of ERK in differentiating TS cells via CB1. (*A*) CB1 is expressed in TS cells. This cell line is stably transfected with the *GFP* gene. Images depict GFP in green, CB1 in red (TS cell surfaces), and the merge in yellow. (Scale bar, 50 μm.) (*B* and *C*) Activation of ERK in TS cells by CP55,940 (CP). TS cells were plated and expanded for 48 h. The cells were serum-starved for 5 h then exposed to different concentrations of CP for 15 min or 7 nM CP for the indicated times or to CP at 7 nM in the presence or absence of a MEK1/2 inhibitor (U0126), CB1-selective antagonist SR141716A (SR1), or CB2-selective antagonist SR144528 (SR2) for 5 min. Phosphorylation of ERK1 in differentiating TS cells was rapidly induced by 7 nM CP. U0126 or SR1, but not SR2, inhibited this activation. Quantitative analysis of ERK activation in *C* is expressed as percentage relative to the maximum band intensity.

CB2 antagonist SR144528, inhibited this activation in the presence of 7 nM CP55,940 (Fig. 3 *B* and *C*). These results uphold the similar nature of biphasic effect that was observed for anandamide in blastocysts (Fig. 1 *B* and *C*).

Anandamide at High Concentration Inhibits Depolarization-Induced Ca^{2+} Influx in Dormant Blastocysts via CB1. Because cannabinoid receptors are negatively coupled to voltage-gated Ca^{2+} channels (4), we next investigated whether anandamide at low and high levels differentially influences intracellular Ca^{2+} signaling in blastocysts. We observed that exposure of dormant blastocysts to increased concentration of KCl from 6 to 60 mM rapidly activates Ca^{2+} influx in the blastocyst trophectoderm with a peak between 30 and 60 sec (Fig. 4). This KCl-induced Ca^{2+} mobilization was greatly abolished by chelating extracellular Ca^{2+} with BAPTA (Fig. 4), suggesting that voltage-gated Ca^{2+} channels are present in blastocyst trophectoderm cells. Indeed, the presence of immunoreactive N- and L-type, but not P/Q-type, Ca^{2+} channels was detected in the blastocyst trophectoderm cells (Fig. 5*A*). We observed that anandamide at 28 nM dramatically inhibited KCl-induced Ca^{2+} influx, whereas anandamide at 7 nM failed to exhibit such an effect (Fig. 5*B*). In addition, this inhibition by 28 nM anandamide was specifically reversed by the CB1-selective antagonist SR141716A, but not by the CB2-selective antagonist SR144528 (Fig. 5*B*), suggesting that this effect of anandamide is mediated by blastocyst CB1. Anandamide by itself at a low or high dose did not alter Ca^{2+} mobilization in the absence of increased KCl concentration (data not shown). ω-Conotoxin GVIA, a known blocker of N-type Ca^{2+} channel, dramatically inhibited KCl-induced Ca^{2+} influx, whereas Calciseptine, a known blocker of L-type Ca^{2+} channel, was less effective (Fig. 6*A*). These results, along with the observation of the failure of (+/−)-Bay K 8644, a specific activator of L-type Ca^{2+} channel, to significantly facilitate Ca^{2+} mobilization (Fig. 6*B*), suggest that anandamide primarily influences N-type Ca^{2+} channels in the blastocyst trophectoderm. This is consistent with previous observations that Ca^{2+} signaling is crucial for blastocyst formation (28) and that N-type Ca^{2+} channels are functionally coupled to the stimulating effects of heparin-binding EGF-like growth factor (HB-EGF) in blastocyst development and outgrowth (22).

Anandamide at Low Concentration Confers Blastocyst Competency to Implantation via CB1. The present observation of ERK activation and inhibition of Ca^{2+} mobilization, coupled with our previous findings of stimulation and inhibition of trophoblast growth and implantation at low and high cannabinoid levels (11–13), respectively, suggests that cannabinoid-induced stimulatory and inhibitory influences on blastocyst functions and implantation are executed by different signal transduction pathways, such as the ERK and Ca^{2+} signaling pathways. To obtain further insights into the significance of these observations, we used physiologically relevant models of embryo transfer and delayed implantation. Preimplantation ovarian estrogen secretion on day 4 of pregnancy is essential for implantation. This process is deferred if ovaries are removed before the preimplantation estrogen secretion, resulting in delayed implantation that can be main-

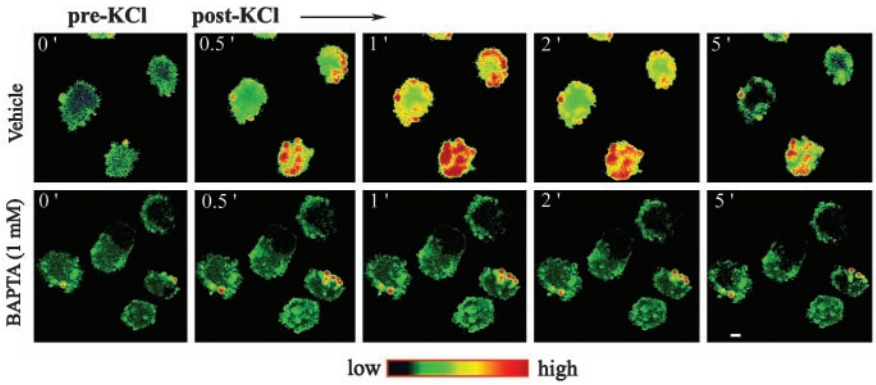

Fig. 4. Depolarization-induced Ca^{2+} mobilization in dormant blastocysts. Exposure of dormant blastocysts to increased concentration of KCl from 6 to 60 mM rapidly activates voltage-gated Ca^{2+} channels in the blastocyst trophectoderm, showing a peak between 30 and 60 sec. Chelating extracellular Ca^{2+} with 1 mM 1,2-bis(o-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetate (BAPTA) abolished this influx. The relative level of intracellular Ca^{2+} is indicated by the fluorescent intensity, which is displayed in pseudocolor according to the color bar by using the program LSMIB (Zeiss). (Scale bar, 20 μm.)

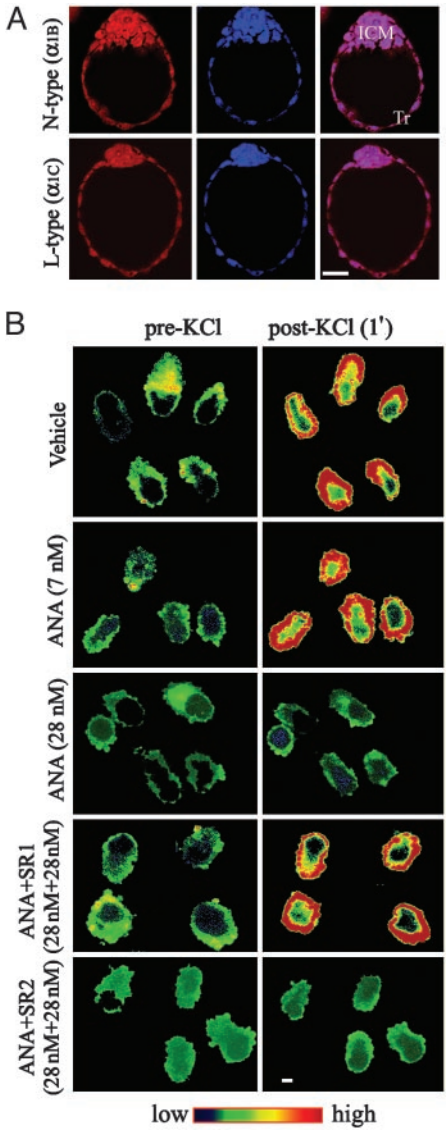

Fig. 5. Inhibition of depolarization-induced Ca^{2+} influx by 28 nM anandamide in dormant blastocysts. (*A*) Distribution of Ca^{2+} channel α subunits, α_{1B} (N-type) and α_{1C} (L-type), in dormant blastocysts. Both inner cell mass (ICM) and trophectoderm cell (Tr) are decorated with α_{1B} and α_{1C} subunits shown in red, Hoechst-labeled nuclei in blue, and the merge in pink. (*B*) Inhibition of depolarization-induced Ca^{2+} influx by anandamide (ANA). Ca^{2+} mobilization in blastocysts was visualized with Fluo-4 acetoxymethyl ester. Depolarization-induced Ca^{2+} influx in dormant blastocysts after exposure to 60 mM KCl was dramatically inhibited by 28 nM anandamide but not by 7 nM. The CB1-selective antagonist SR141716A (SR1) at equimolar concentration reversed this inhibition, but the CB2-selective antagonist SR144528 (SR2) was ineffective. The relative level of intracellular Ca^{2+} is indicated by the fluorescent intensity, which is displayed in pseudocolor according to the color bar by using LSMIB. (Scale bar, 20 μm.)

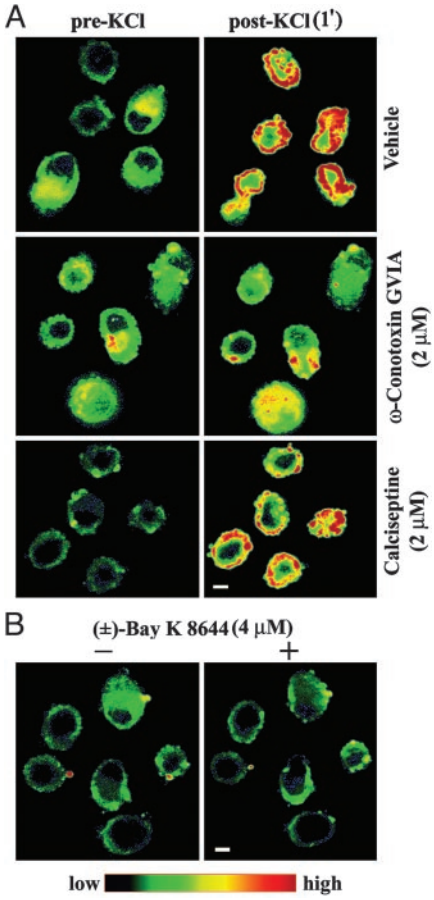

Fig. 6. Identification of Ca^{2+} channel subtypes in dormant blastocysts. (*A*) ω-Conotoxin GVIA (2 μM), a selective blocker of N-type Ca^{2+} channel, dramatically inhibited KCl-induced Ca^{2+} influx, whereas an equimolar concentration of Calciseptine, a selective blocker of L-type Ca^{2+} channel, was less effective. (*B*) Furthermore, (+/−)-Bay K 8644 (4 μM), a selective activator of L-type Ca^{2+} channels, failed to influence Ca^{2+} mobilization. These results suggest that the N-type Ca^{2+} channel is more active in the dormant blastocyst. The relative level of intracellular Ca^{2+} is indicated by the fluorescent intensity in pseudocolor. (Scale bar, 20 μm.)

tained for many days by continued P_4 treatment with the uterus remaining in a quiescent state and blastocysts in dormancy (18, 26, 27). Dormant blastocysts, if cultured *in vitro*, gain metabolic competence (29). However, we have previously shown that metabolic competency alone fails to confer implantation competency to dormant blastocysts; the gain of implantation competency is also an important factor in the initiation of implantation in the receptive uterus (19). We show here that dormant blastocysts exposed to 7 nM anandamide, but not 28 nM, become implantation-competent. For example, dormant blastocysts cultured for 24 h in the presence or absence of 7 or 28 nM anandamide were transferred into uterine lumens of P_4-treated recipients 4 h after an injection of E_2 (3 ng per mouse). Under these conditions, activated blastocysts do implant, whereas dormant blastocysts fail to implant (18, 19). A large number of blastocysts exposed to 7 nM anandamide showed implantation, whereas those exposed to the vehicle or 28 nM anandamide failed to implant in similarly treated uteri (Table 1), but no sign of cell death at 28 nM anandamide was noted. A CB1-selective inhibitor SR141716A or a MEK1/2 inhibitor U0126 blocked the activation of dormant blastocysts for implantation by 7 nM anandamide (data not shown). These results suggest that blastocysts in culture remain implantation-incompetent but gain implantation competency at a lower anandamide concentration in the medium.

To provide genetic confirmation that the effects of anandamide on blastocyst activation are mediated via CB1, $CB1^{-/-}$ dormant blastocysts were cultured for 24 h either in the presence of the vehicle alone or 7 nM anandamide. We observed that most of the $CB1^{-/-}$ dormant blastocysts remained incompetent to implantation in response to 7 nM anandamide (Table 1). These results provide genetic confirmation that CB1 in the blastocyst

Table 1. Anandamide at 7 nM confers blastocyst competency to implantation via CB1

Genotype	Treatment	No. of recipients	No. of blastocysts transferred	No. of mice with IS	No. of IS (%)	No. of blastocysts recovered
WT	Vehicle	5	57	0	0	3
	ANA (7 nM)	10	112	10	55 (49%)*	NA
	ANA (28 nM)	5	63	1	1 (2%)	14
CB1$^{-/-}$	Vehicle	5	72	3	4 (6%)	16
	ANA (7 nM)	5	61	3	4 (7%)	6

WT or CB1 mutant dormant blastocysts were cultured for 24 h in the presence of the vehicle (control) or 7 or 28 nM anandamide (ANA). After culture, blastocysts were transferred into uterine lumens of P_4-primed delayed implanting pseudopregnant WT recipient 4 h after an injection of E_2 (3 ng per mouse). Mice were examined for implantation sites (IS) 24 h later. If no implantation site was noted, uteri were flushed to recover blastocysts. Implantation rate was significantly higher for WT dormant blastocysts cultured with 7 nM anandamide (*, $P < 0.01$, unpaired *t* test). NA, not applicable.

interacts with endocannabinoids in a concentration-dependent manner with respect to embryo development and implantation.

The "window" of implantation is defined as the time during which the activated stage of the blastocyst is superimposed on the receptive state of the uterus (19). Our genetic, pharmacological, and physiological analyses show that anandamide at a low concentration activates ERK signaling in dormant blastocysts via their trophectoderm cell surface CB1 and renders them competent for implantation in the receptive uterus. In contrast, failure to achieve ERK activation and implantation competency in blastocysts with the inhibition of Ca^{2+} mobilization at a higher anandamide level provides evidence that elevated cannabinoid levels are detrimental to blastocyst activation and implantation (Fig. 7, which is published as supporting information on the PNAS web site). This is consistent with the finding that elevated levels of cannabinoids inhibit implantation in WT mice but not in *CB1*$^{-/-} \times$ *CB2*$^{-/-}$ double-mutant mice (11). These results are relevant to humans because spontaneous pregnancy losses occur in women with elevated anandamide levels (30, 31). Thus, regulated endocannabinoid signaling is at least one of the pathways that determine the fate of embryo implantation. Our present observation of differential regulation of ERK and Ca^{2+} signaling by anandamide within a very narrow range provides for the first time a potential "cannabinoid sensor" mechanism for influencing crucial steps during early pregnancy. We propose that critical levels of uterine endocannabinoids interact with appropriately expressed blastocyst CB1 in synchronizing blastocyst activation with uterine receptivity for implantation, whereas aberrant levels of uterine endocannabinoids and/or blastocyst CB1 interfere with these processes, resulting in pregnancy termination. In conclusion, this study places the embryo as a target for natural and endocannabinoids and raises the significance of cannabinoid signaling in female fertility.

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