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The preimplantation mouse embryo is a target for cannabinoid ligand-receptor signaling

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ABSTRACT **Using a reverse transcription-coupled PCR, we demonstrated that both brain and spleen type cannabinoid' receptor (CB1-R and CB2-R, respectively) mRNAs are expressed in the preimplantation mouse embryo. The CB1-R mRNA expression was coincident with the activation of the embryonic genome late in the two-cell stage, whereas the CB2-R mRNA was present from the one-cell through the blastocyst stages. The major psychoactive component of marijuana (−)-Δ-9-tetrahydrocannabinol [(-)-THC] inhibited forskolin-stimulated cAMP generation in the blastocyst, and this inhibition was prevented by pertussis toxin. However, the inactive cannabinoid cannabidiol (CBD) failed to influence this response. These results suggest that cannabinoid receptors in the embryo are coupled to inhibitory guanine nucleotide binding proteins. Further, the oviduct and uterus exhibited the enzymatic capacity to synthesize the putative endogenous cannabinoid ligand arachidonylethanolamide (anandamide). Synthetic and natural cannabinoid agonists [WIN 55,212-2, CP 55,940, (-)-THC, and anandamide], but not CBD or arachidonic acid, arrested the development of two-cell embryos primarily between the four-cell and eight-cell stages *in vitro* in a dose-dependent manner. Anandamide also interfered with the development of eight-cell embryos to blastocysts in culture. The autoradiographic studies readily detected binding of [^{3}H]anandamide in embryos at all stages of development. Positive signals were present in one-cell embryos and all blastomeres of two-cell through four-cell embryos. However, most of the binding sites in eight-cell embryos and morulae were present in the outer cells. In the blastdcyst, these signals were primarily localized in the mural trophectoderm with low levels of signals in the polar trophectoderm, while little or no signals were noted in inner cell mass cells. These results establish that the preimplantation mouse embryo is a target for cannabinoid ligands. Consequently, many of the adverse effects of cannabinoids observed during pregnancy could be mediated via these cannabinoid receptors. Although the physiological significance of the cannabinoid ligand-receptor signaling in normal preimplantation embryo development is not yet clear, the regulation of embryonic cAMP and/or Ca^{2+} levels via this signaling pathway may be important for normal embryonic development and/or implantation.**

Marijuana and its cannabinoid derivatives have been used for thousands of years as psychoactive agents. Cannabinoids exert a wide spectrum of central and peripheral effects including psychotropic, hypnotic, tranquilizing, antiemetic, anticonvulsive, and analgesic effects. They can also lower' intraocular pressure, increase appetite, and affect cardiovascular, reproductive, and immune systems (1). One concern regarding exposure to cannabinoids is their apparent adverse effects on embryonic development and pregnancy (2-5). However, the mechanisms by which cannabinoids exert these diverse effects were not clearly understood. The recent identification and cloning of inhibitory guanine nucleotide binding protein (G protein) (G_i)-coupled cannabinoid receptors in the brain (CB1-R) and spleen (CB2-R) provide evidence that many of these effects could be mediated via these receptors (6-8). The identification of a putative endogenous cannabinoid ligand, anandamide (arachidonylethanolamide), in the brain further suggests that cannabinoid ligand-receptor signaling could be operative in the central nervous system (9, 10), though its physiological significance is not yet understood. Sporadic reports of adverse effects of cannabinoid exposure on embryonic growth and development in several species (2-5) prompted us to examine whether the preimplantation mouse embryo expresses functional cannabinoid receptors, whether cannabinoid agonists influence their development in *vitro*, and whether the reproductive tract has the capacity to synthesize anandamide.

MATERIALS AND METHODS

Animals. CD-l female mice (48 days old; Charles River Breeding Laboratories) were mated with males of the same strain to induce pregnancy (day 1 = vaginal plug discovery). Embryos at different stages of development were recovered in Whitten's medium by flushing the reproductive tract (11, 12).

Analysis of CB1-R and CB2-R mRNAs. To examine CBl-R or CB2-R mRNA expression in the preimplantation mouse embryo, reverse transcription-coupled PCR (RT-PCR) was employed (13). For RT-PCR analysis, the following primers were used: 5′-GGAGAACATCCAGTGTGGGG-3′ (sense) and 5'-CATTGGGGCTGTCTTTACGG-3' (antisense) for the CBl-R transcript (7), and 5′-CCTGTTGAAGATCGGCAGCG-3' (sense) and 5'-GGTAGGAGATCAACGCCGAG-3' (antisense) for the CB2-R transcript (8). The internal oligonucleotides 5′-GGTTCTGGAGAACCTACTGG-3′ and 5′-TGGGCAGCCTGCTGCTGACT-3' were used for Southern blot hybridization of the amplified products for CBl-R and CB2-R, respectively. The β-actin sense and antisense primers designed from the mouse β-actin cDNA were 5′-GTGGGCCGCTCTAGGCACCAA-3' and 5′-CTCTTTGATGTCACGCACGATTTC-3′, respectively (14). An internal oligonucleotide 5′-CCACGGGCATTGTGATGGAC-3′ was used for Southern blot analysis. RNA from the brain, spleen, uterus, or 70-80 embryos was isolated (13). Total RNA (1 μg) from the brain, spleen, or uterus, or 25% of the embryonic RNA was reverse-transcribed by using specific antisense primers. One-third of the RT products was PCR-amplified using the sense and antisense primers as described (13). PCR cycle parameters were as follows: 94°C for 4 min, 55°C for 1.5 min, and 72°C for 2.5 min for the first cycle followed by 94°C for 1 min, 55°C for 1.5 min, and 72°C for 2.5 min for 35 cycles. One-tenth of the

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Abbreviations: CBl-R and CB2-R, brain and spleen type cannabinoid receptors, respectively; THC, A-9-tetrahydrocannabinol; CBD, cannabidiol; G protein, guanine nucleotide binding protein; G_i, inhibitory G protein; RT-PCR, reverse transcription-coupled PCR; ICM, inner cell mass.

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amplified product was electrophoresed on agarose gels (1.5%), stained with ethidium bromide, and analyzed by Southern blot hybridization. Experimental and negative controls were run simultaneously.

Analysis of Forskolin-Stimulated cAMP Accumulation. Blastocysts in a batch of 35-50 were sonicated in 20 μl of 50 mM Tris·HCl, pH 7.5 / 1 mM EGTA/0.5 mM 3-isobutyl-1-methylxanthine. The blastocyst homogenates were incubated in 100 μl of the same buffer containing 10 μM GTP, 1 mM ATP, 3 mM $MgSO_4$, and ±5 μM forskolin at 25°C for 10 min to assess cAMP accumulation. To determine whether the psychoactive cannabinoid (−)-Δ-9-tetrahydrocannabinol [(−)-THC] or the inactive cannabidiol (CBD) can influence forskolin-stimulated cAMP accumulation, THC (10 μM) or CBD (10 μM) was added to the incubation mixture with forskolin (5 μM). To determine that the THC effects were mediated via G proteins (G_i), blastocyst homogenates were preincubated with pertussis toxin (5 ng/ml) for 5 min prior to the addition of forskolin and THC. The reactions were terminated by boiling for 5 min and centrifuged. cAMP in the supernatants was measured by a protein binding assay as described (15, 16).

Autoradiographic Detection of Ligand Binding Sites. Autoradiographic detection of ligand binding sites using [^{3}H]anandamide was performed in preimplantation embryos (11, 13). Inner cell mass (ICM) cells were isolated from blastocysts by immunosurgery (17). Embryos and ICMs were incubated with 4.5 nM [^{3}H]anandamide (specific activity, 221 Ci/mmol; 1 Ci = 37 GBq; DuPont/NEN) in Whitten's medium containing 0.3% bovine serum albumin in the absence or presence of 500-fold molar excess of unlabeled cannabinoid agonists [anandamide, (-)-THC, WIN 55,212-2, or CP 55,940] or inactive cannabinoids [CBD or (+)-THC] for 1 h at 37°C. After incubation, embryos and ICMs were washed six times in the same medium at 4°C, fixed in 2% (wt/vol) paraformaldehyde in phosphate-buffered saline, centrifuged onto glass slides, air-dried, and subjected to autoradiography for 14 h (11, 13). Embryos and ICMs were poststained with hematoxylin and examined under a microscope. Autoradiographic signals in the blast'ocyst were also examined under a confocal microscope. Bright-field photomicrographs at X400 magnification are shown. Black grains indicate the sites of anandamide binding.

Enzymatic Synthesis of Anandamide. The enzymatic synthesis of anandamide was determined as described (18-20). Oviduct, uterine, or brain tissues were homogenized in ice-cold TE buffer (10 mM Tris·HCl/1 mM EDTA, pH 7.6) containing 1.5 mM phenylmethylsulfonyl fluoride and centrifuged at 2000 X *g* for 15 min. The supernatants '(300 μg of protein) were incubated in 1 ml of 0.1 M Tris·HCl, pH 9.0/1.5 mM phenylmethylsulfonyl fluoride/1 μCi of [5,6,8,9,11,12,14,15-^{3}H (N)]arachidonic acid (specific activity, 221 Ci/mmol; DuPont/NEN)/±7 mM ethanolamine at 37°C for 1 h. The reaction mixtures were extracted by 2 ml of chloroform/methanol, 1:1 (vol/vol). The organic phase was removed, dried under nitrogen gas, and then dissolved in 10 μl of chloroform/methanol. The samples were spotted onto silica gel-coated plates and run parallel with ^{3}H-labeled standards, anandamide (DuPont/NEN) and arachidonic acid. TLC was performed as described (28). The plate was sprayed with EN^{3}HANCE (DuPont/NEN) and exposed to an x-ray film at -70°C for l-2 days. After autoradiography, spots corresponding to anandamide were scraped, and radioactivity in them was measured.

Culture of Preimplantation Embryos. To study the effects of cannabinoid agonists on preimplantation embryo development, two-cell embryos were recovered on day 2 (0830-0900 h), pooled in Whitten's medium containing 0.3% bovine serum albumin (11, 12), washed four times in the same 'medium, and cultured in groups of 5-10 in 25 μl of Whitten's medium under silicon oil in an atmosphere of 5% CO_2/95% air at 37°C for 72 h with various agonists as indicated in Fig. 6. The agonists used were (-)-THC (National Institute on Drug Abuse, Research Triangle Park, NC), CP 55,940 (Pfizer Diagnostics), WIN 55,212-2, and anandamide (Research Biochemicals, Natick, MA). CP 55,940 is a synthetic THC analogue, and WIN 55,212-2 is a synthetic noncannabinoid aminoalkylindole that binds with cannabinoid receptors (21). CBD (National Institute on Drug Abuse) or arachidonic acid (Sigma) was used as a control for (-)-THC or anandamide, respectively. Eight-cell embryos recovered at 0830–0900 h on day 3 were similarly cultured in the presence of anandamide (3.5 and 7 nM) for 24 h. All test agents were dissolved in ethanol and diluted with Whitten's medium. The final ethanol concentration was <0.1%. The control cultures contained the same concentration of ethanol. The test agents were added at the beginning of culture. The embryos were observed every 12 h to monitor their development. At termination of culture, the number of embryos that formed blastocysts was recorded, and those that did not form blastocysts were examined to determine the stage at which their development was arrested. Embryos that developed to blastocysts were subjected to differential cell counts after termination of the culture (17). In some experiments, embryos were also cultured in fresh medium after exposure to anandamide for 24 h to determine whether the effects were reversible.

RESULTS

Cannabinoid Receptor mRNAs in the Preimplantation Mouse Embryo. As reported for the rat (7, 8), RT-PCR detected CBl-R mRNA in the mouse brain and CB2-R mRNA in the spleen (Fig. 1). In the embryo, CBl-R mRNA was primarily detected from the four-cell through the blastocyst stages (Fig. 1*A*), whereas CB2-R mRNA was present from the one-cell through the blastocyst stages (Fig. 1*B*). RNA integrity was confirmed by the detection of β-actin mRNA in these samples. Quantitative PCR is required to determine more precisely the developmental changes in these mRNA levels.

Anandamide Binding Sites in the Blastocyst. Numerous binding sites for [^{3}H]anandamide were evident within a short period (14 h) of autoradiographic exposure. Unlabeled cannabinoid agonists, but'not the inactive cannabinoids, competed for this binding. In one-cell through four-cell embryos, the

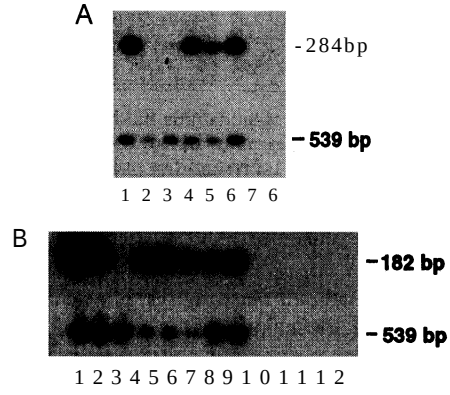

FIG. 1. Analysis of CBl-R and CB2-R transcripts in the preimplantation mouse embryo. (*A*) Southern blot analysis of RT–PCR-amplified products of CBl-R (284 bp) or β-actin (539 bp). Lanes: 1, mouse brain; 2-6, embryos at one-cell, two-cell, four-cell, eight-cell/morula, and blastocyst stages, respectively; 7, mouse brain RNA without RT reaction; 8, primer control. (*B*) Southern blot analysis of RT-PCR-amplified products of CB2-R (182 bp) or β-actin (539 bp). Lanes: 1, rat spleen; 2, mouse spleen; 3, day 1 pregnant uterus; 4-8, embryos at one-cell, two-cell, four-cell, eight-cell/morula, and blastocyst stages, respectively; 9-11, rat spleen, mouse spleen, and mouse blastocyst RNA without RT reaction, respectively; 12, primer control. These experiments were performed twice with similar results.

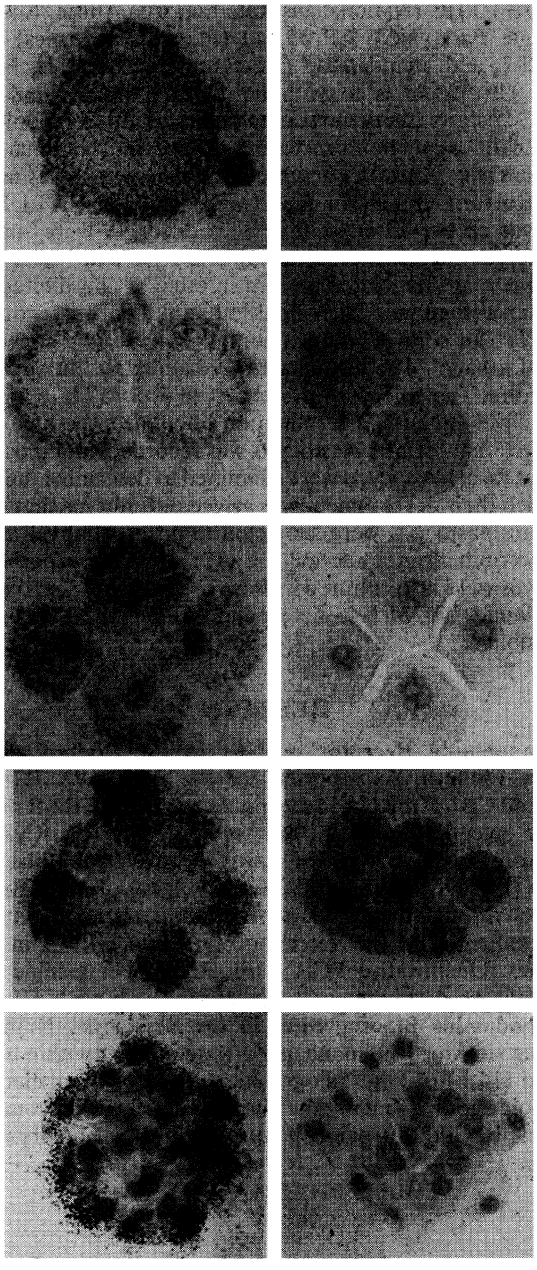

FIG. 2. Autoradiographic anandamide binding sites in the blastocyst. Bright-field photomicrographs are at x 260. Autoradiographic binding sites (black grains) in representative one-cell embryos through morulae. (Left) Binding sites in the presence of [^{3}H]anandamide. *(Right)* Nonspecific binding sites in the presence of 500-fold molar excess of unlabeled anandamide.

signals were present in all blastomeres. However, in eight-cell embryos or morulae, the majoritv of the binding sites were noted in the outer cells (Fig. 2). In the blastocyst, the binding sites were primarily localized in the mural trophectoderm with low intensity signals in the polar trophectoderm, but little or no signal was present in the ICM cells (Fig. 3). This was confirmed by the results obtained with the isolated ICMs (data not shown) and by three-dimensional projection of confocal images of intact blastocysts by using reflected light optics (Fig. 3).

Effects of Cannabinoids on the Forskolin-Stimulated cAMP Accumulation in the Blastocyst. In the brain, the cannabinoid receptor is a member of the G-protein-coupled superfamily and inhibits adenylyl cyclase and N-type Ca^{2+} channel (6, 7). To examine whether the embryonic cannabinoid receptors were coupled to G proteins (G_i), the effects of (-)-THC or CBD on forskolin-stimulated cAMP accumulation in blastocyst homogenates were measured with or without pertussis toxin pretreatment. (-)-THC, but not CBD, inhibited forskolin-stimulated cAMP accumulation and this inhibition was prevented by pertussis toxin pretreatment (Fig. 4). This suggests that cannabinoid receptors in the blastocyst are coupled to G_i and the response is specific to the active cannabinoid.

Synthesis of Anandamide in the Reproductive Tract. To examine whether the reproductive tract, like the brain, could be a source of the endogenous ligand, mouse oviduct, uterine, or brain homogenates were incubated with labeled arachidonic acid in the presence or absence of ethanolamine to determine the enzymatic synthesis of anandamide (18-20). As shown in Fig. 5, both the oviduct and uterus have the capacity to synthesize anandamide in the presence of ethanolamine. However, the synthesizing capacity of the oviduct appears to be lower than that of the brain or uterus-e.g., the conversion of [^{3}H]arachidonic acid to anandamide was ≈3% for the oviduct and ≈7% for the brain or uterus.

Effects of Cannabinoid Agonists on Preimplantation Embryo Development. To examine whether cannabinoid ligands influence embryo development, two-cell embryos were cultured in the presence or absence of synthetic or natural cannabinoid agonists (11). All agonists [(-)-THC, CP 55,940, Win 55212-2, and anandamide] exhibited dose-dependent inhibition of embryonic development to blastocysts (Fig. 6). As shown in other systems (7, 8), THC was relatively less potent than the other cannabinoid agonists in this response. The developmental arrest primarily occurred between the four-cell and eight-cell stages. Further, embryos arrested at the eight-cell stage did not exhibit compaction, which occurs during normal development. CBD or arachidonic acid, used as a control for (-)-THC or anandamide, respectively, did not alter embryonic development significantly (Fig. 6). Results of differential cell counts showed a reduction in trophectoderm cell numbers in the anandamide-treated (3.5 nM) embryos that reached the blastocyst stage compared with the controls (19.5 ± 1.0 cells, n = 13, vs. 26.0 ± 1.0 cells, n = 18; $P < 0.001$, Student's t test). The ICM cell number did not change between the treated and control blastocysts (8.0 ± 0.9 cells, n = 13, vs. 9.0 ± 0.6 cells, n = 18). The failure of CBD or arachidonic acid to interfere with embryonic development suggests that the effects of cannabinoid agonists on embryo development were

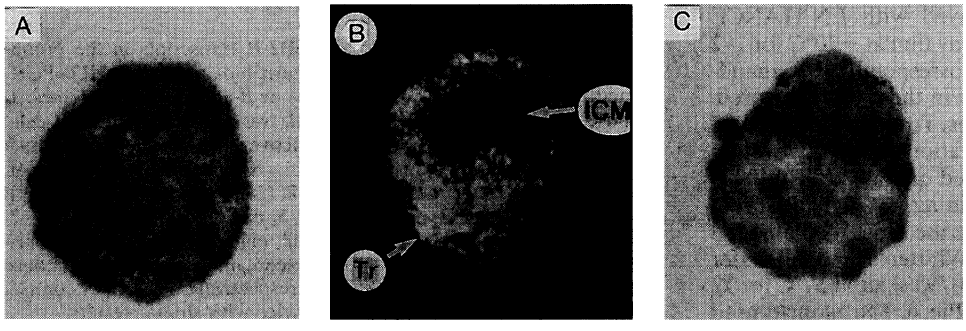

FIG. 3. Autoradiographic binding sites in representative blastocysts. (*A*) Binding sites (bright field; black grains) in the presence of [^{3}H]anandamide. (*B*) Confocal microscopy of *A* (white grains). (C) Nonspecific binding (bright field) in the presence of 500-fold molar excess of unlabeled anandamide. Unlabeled (-)-THC, WIN 55212-2, or CP 55,940, but not (+)-THC or CBD, also competed for [^{3}H]anandamide binding (data not shown). Tr, trophectoderm.

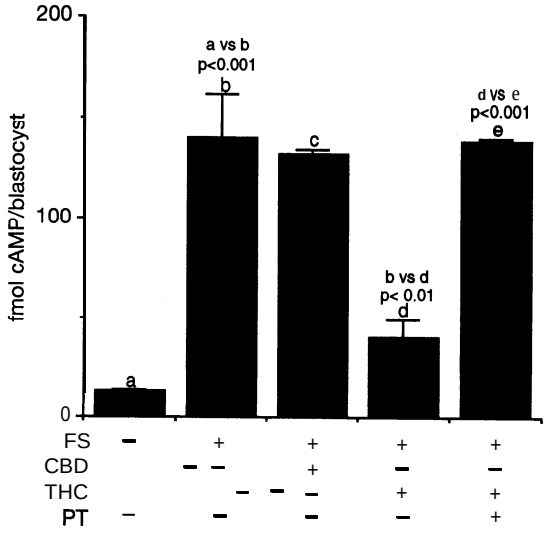

FIG. 4. Effects of cannabinoid agonists on forskolin-stimulated cAMP accumulation in the preimplantation embryo. FS, forskolin; PT, pertussis toxin. Results are the mean ± SD of triplicate experiments. Statistical comparisons (*t* test) are shown.

specific and not due to nonspecific toxic effects. This suggestion is consistent with the observation that a considerable number of embryos developed to the eight-cell stage after 48 h of culture with these agonists. Furthermore, even at the highest concentration used, there was never a complete arrest of embryonic development (Fig. 6), and a large number of the embryos (69%) recultured in fresh medium 24 h after exposure to anandamide (14 nM) developed into blastocysts. About 37% and 53% of the eight-cell embryos cultured for 24 h in the presence of 3.5 and 7.0 nM anandamide, respectively, failed to

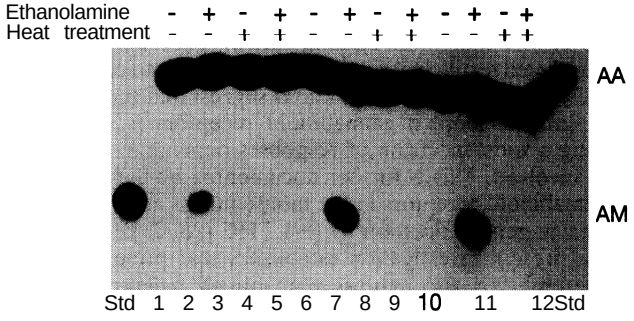

FIG. 5. Enzymatic synthesis of anandamide. Lanes: 1-4, day 4 pregnant oviduct; 5-8, day 4 pregnant uterus; 9-12, brain. Standards (Std) of [^{3}H]arachidonic acid (AA) and [^{3}H]anandamide (AM) are shown. These experiments were repeated twice with similar results. Note that no reaction products were obtained in the absence of ethanolamine or when heat-treated tissue extracts were used.

develop into blastocysts. At 7.0 nM, but not at 3.5 nM, of anandamide, blastocysts that developed in culture from eight-cell embryos again showed a reduction in the number of trophectoderm cells as compared to controls (15.0 ± 0.6 cells, n = 17 vs. 20.0 ± 1.0 cells, n = 16; $P < 0.001$, Student's t test). No significant changes were noted in ICM cell numbers between the treated and control blastocysts (9.0 ± 0.7 cells, n = 17, vs. 10.0 ± 0.2 cells, n = 16).

DISCUSSION

The cannabinoid receptors expressed in the brain are now thought to be the mediators of many of the central effects exerted by cannabinoids, while the receptors expressed in the spleen and leukocytes are associated with the antiinflammatory and immunosuppressive effects of these agonists (22-24).

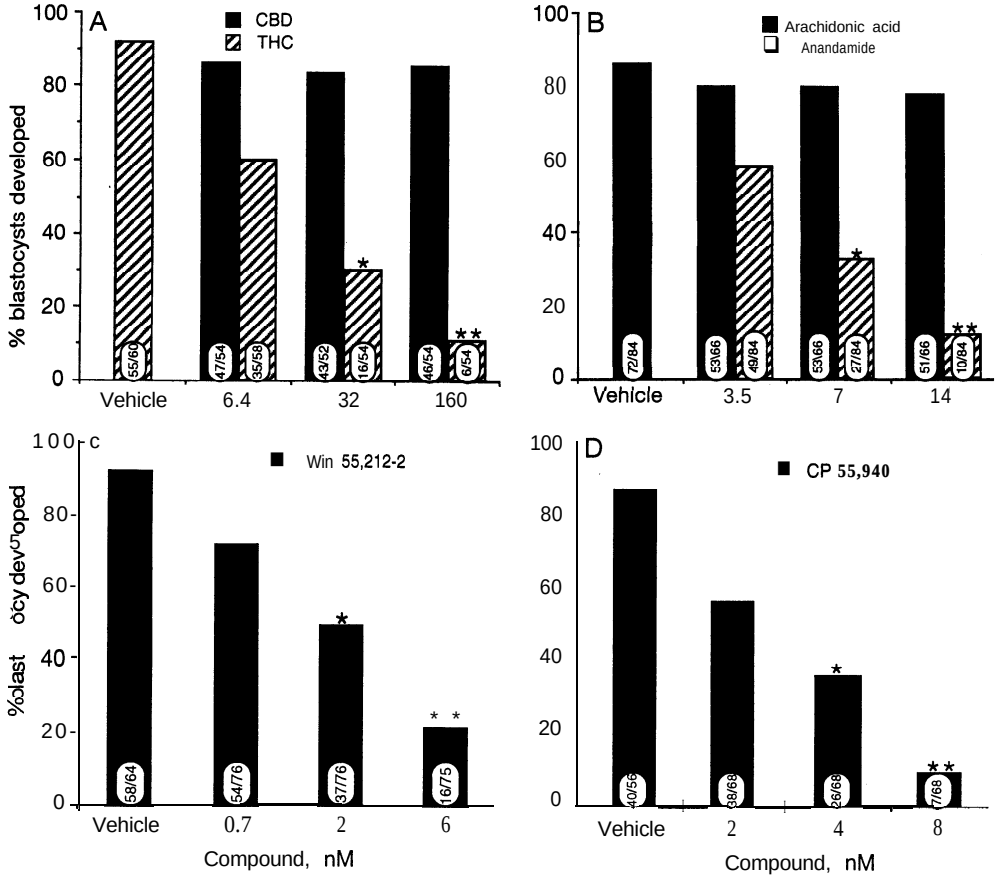

FIG. 6. Effects of cannabinoid agonists on preimplantation embryo development. Effects of THC and CBD (A), anandamide and arachidonic acid *(B),* WIN 55,212-2 (*C*), and CP 55,940 (*D*). The numbers within the bars indicate the number of blastocysts that developed/total number of two-cell embryos cultured. Each experiment was repeated five or six times with controls run simultaneously (statistical analysis by χ^2 and Fisher exact tests; *, $P < 0.05$; **, $P <$ 0.001 when compared to controls).

The expression of cannabinoid receptors in the testis and the presence of cannabinoid binding sites in sperm correlate with their reduced fertilizing capacity after exposure to cannabinoid ligands (25-27). These observations suggest that the activation of G_i-protein-coupled cannabinoid receptors is capable of evoking a wide spectrum of responses depending on the cell types involved. This is further documented by the expression of cannabinoid receptors in the mouse uterus and modulation of uterine gene expression by (-)-THC (28, 29).

The present investigation establishes that preimplantation mouse embryos also express cannabinoid receptors and respond to cannabinoid agonists in *vitro*. The differential temporal expression of the CBl-R and CB2-R mRNAs in the preimplantation embryo is an interesting observation, the significance of which is not yet clear. The CB2-R mRNA is apparently of maternal origin and persists through the blastocyst stage, whereas the accumulation of CBl-R mRNA appears to be associated with the activation of the embryonic genome. The presence of cannabinoid ligand binding sites in the embryo throughout the preimplantation period suggests that CB-2 mRNA is also translated in the early stages of development. The overlapping expression of these two receptors in the embryo throughout the preimplantation period suggests that the embryo could be a target for the cannabinoid agonists at any stage during this period. Although cannabinoid ligands can bind to both of these receptors, it is not known whether these receptors behave differently in response to ligand activation during development. The apparent presence of anandamide binding sites in the outer cells of eight-cell embryos or morulae is an interesting observation, the physiological significance of which has yet to be defined. However, it is possible that cannabinoid ligand-receptor signaling at these developmental stages may be associated with the commitment of embryonic cells to the trophectoderm and ICM. Further, the identification of these binding sites in the mural trophectoderm suggests that the activation of this receptor may be involved in the implantation process. Nonetheless, the receptor-expressing cells in the embryo are probably the target for the cannabinoid agonists. Inhibition of forskolin-stimulated cAMP accumulation in the blastocyst by (-)-THC and its prevention by pertussis toxin pretreatment suggest that embryonic cannabinoid receptors are coupled to G_i proteins. In this respect, it should be noted that G_i-like proteins are present in the preimplantation mouse embryo (30). However, the physiological significance of cannabinoid receptors in the preimplantation embryo is not yet clearly understood.

Although the oviduct and uterus have the capacity to synthesize anandamide, it is not known whether this occurs in *vivo*. Recently, an alternative pathway for anandamide synthesis has been identified in the neuronal tissues (31). If this ligand is available to the embryo during its normal development, it may modulate the intracellular concentration of cAMP and/or Ca^{2+} in the embryo. These two second messengers, involved in important signal transduction pathways, are implicated in cell proliferation, differentiation, and gene expression. In this respect, cAMP has been implicated in zygotic gene activation and blastocyst expansion (32, 33), while intracellular Ca^{2+} plays an important role in the cell polarity and embryonic compaction for morula to blastocyst transformation (34, 35). Failure of embryos to proceed beyond the eight-cell stage after exposure to cannabinoid ligands in culture could be due to the inhibition of Ca^{2+} channels resulting from the activation of the cannabinoid receptors. Therefore, tight regulation of the levels of cAMP and Ca^{2+} is likely to be critical for normal embryonic development. Although embryonic arrest after exposure to cannabinoids in *vitro* is consistent with in *vivo* findings of retarded embryonic development and pregnancy failure after chronic exposure to exogenous cannabinoids (2–5), it still cannot be ascertained whether the *in vivo* effects of cannabinoids are mediated via these embryonic receptors or by some other mechanism. Nonetheless, our findings establish the preimplantation embryo as a target for cannabinoid ligand-receptor signaling, raising further concerns with the recent increase in marijuana use among youths (36). Detailed studies regarding the regulation of these receptor genes in the preimplantation embryo and that of the ligand in the reproductive tract are required to gain further insights into the role of cannabinoid ligand-receptor signaling in the preimplantation embryo.

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