Activation of Brain-Type Cannabinoid Receptors Interferes with Preimplantation Mouse Embryo Development

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ABSTRACT

The recent identification and cloning of guanine nucleotide regulatory protein-coupled brain-type and spleen-type cannabinoid receptors (CB1-R and CB2-R, respectively) provide evidence that many of the effects of cannabinoids are mediated via these receptors. Our recent observation of expression of both CB1-R and CB2-R genes in the preimplantation mouse embryo suggests that it could also be a target for cannabinoids. Indeed, cannabinoid agonists interfered with preimplantation embryo development in vitro. To examine whether cannabinoid effects on preimplantation embryos are mediated via CB1-R, we developed rabbit antipeptide antibodies against the N-terminal region of CB1-R and examined the receptor protein in the blastocyst by Western blotting and its spatiotemporal distribution in preimplantation mouse embryos by immunohistochemistry. Cannabinoid binding sites in the blastocyst were examined by Scatchard analysis, while the reversibility of cannabinoid-induced embryonic arrest in vitro was monitored using a specific antagonist to CB1-R, SR141716A. Western blot analysis detected a major band of ~59 kDa and a minor band of ~54 kDa in the blastocyst. Immunochemistry detected this receptor protein from the 2-cell through the blastocyst stages. Scatchard analysis using [3H]-anandamide (an endogenous ligand) showed a single class of binding sites in Day 4 blastocysts with an apparent $K_d$ of 1.0 nM and $B_{max}$ of 0.09 fmol/blastocyst. Considering the total number of cells (~50) and total protein content (~20 ng) of a blastocyst, it is apparent that the mouse blastocyst has many high-affinity receptors, as those in the mouse brain ($K_d$ 1.8 nM and $B_{max}$ 18.8 pmol/mg membrane protein). Cannabinoid agonists and the CB1-R antagonist SR141716A effectively competed for anandamide binding in the blastocyst. To determine whether cannabinoid inhibition of embryonic development could be reversed by SR141716A, 2-cell embryos were cultured in the presence of cannabinoid agonists with or without SR141716A for 72 h. Most of the 2-cell embryos cultured in the absence of the agonists developed into blastocysts (~90%). In contrast, the addition of cannabinoid agonists anandamide, Win 55212–2, or CP 55,940 in the culture medium severely compromised embryonic development: more than 60% of the 2-cell embryos failed to develop to blastocysts. A reduction in trophoblast cell numbers was noted in those blastocysts that escaped the developmental arrest in the presence of cannabinoid agonists. However, this reduction was corrected when embryos were cultured simultaneously with an agonist and SR141716A. Furthermore, embryonic arrest was reversed when embryos were cultured simultaneously in the presence of an agonist and SR141716A. The addition of SR141716A alone in the culture medium apparently had no effects on embryonic development; more than 90% of the embryos developed into blastocysts. The results suggest that the CB1 receptors in preimplantation mouse embryos are biologically active and cannabinoid effects on them are primarily mediated by these receptors.

INTRODUCTION

Cannabinoid derivatives of marijuana exert a wide spectrum of central and peripheral effects including psychotropic, hypnic, tranquilizing, antinociceptive, psychoactive, and analgesic effects. They can also affect appetite, intraocular pressure in glaucoma, cardiovascular, reproductive, and immune functions [1,2]. One of the major concerns of exposure to cannabinoid derivatives is their reported adverse effects on reproductive functions, including reduced fertilizing capacity of sperm (reviewed in [3–6]). The mechanisms by which cannabinoid effects are mediated were poorly understood until the recent identification and cloning of inhibitory guanine nucleotide binding protein (G$i$)-coupled brain-type and spleen-type cannabinoid receptors (CB1-R and CB2-R, respectively) [7–9]. It is now evident that many of the central and peripheral cannabinoid effects are mediated via these receptors. The CB2-R gene is expressed primarily in the spleen, while that of the CB1-R is expressed predominantly in the brain [8,9]. Furthermore, an endogenous cannabinomimetic lipid derivative, N-arachidonoyl ethanolamide, known as anandamide, has been isolated from the porcine brain [10,11]. This compound binds with high affinity to brain-type and spleen-type cannabinoid receptors and mimics most of the effects of Δ$^9$-tetrahydrocannabinol ((−)THC), a psychoactive derivative of marijuana.

The other tissues that express CB1-R are the testis, spleen, and peripheral blood leukocytes [12–14]. The expression of cannabinoid receptors in the spleen and leukocytes is associated with the anti-inflammatory and immunosuppressive roles of cannabinoids [13,14], while the observation of reduced fertilizing capacity of sperm exposed to cannabinoids is consistent with the detection of CB1-R in the testis and sperm [5,6,12]. We have recently demonstrated that both the CB1-R and CB2-R mRNAs are expressed in preimplantation mouse embryos. Further, cannabinoids inhibited forskolin-stimulated cAMP formation in the blastocyst, and this inhibition was prevented by pertussis toxin pretreatment [15]. This suggested that cannabinoid receptors in the mouse embryo, like those in the brain [7,8], are coupled to G$i$ proteins. Furthermore, the mouse oviduct and uterus have the capacity to synthesize the endogenous cannabinoid ligand anandamide [15,16]. These findings suggest that preimplantation mouse embryos are also possible targets for cannabinoid ligand-receptor signaling. Indeed, synthetic and natural cannabinoid agonists (WIN 55212–2, CP 55,940, (−)THC, and anandamide) arrested the development of 2-cell embryos into blas-
to cysts in vitro [15]. Although embryonic arrest appeared to be specific to cannabinoid agonists, it was not known whether the inhibitory effects were mediated via CB1-R or CB2-R. In the study reported here, we addressed this issue using antipeptide antibodies to CB1-R, and SR141716A [N-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole-carboxamide hydrochloride], a potent and specific antagonist to CB1-R [17, 18].

MATERIALS AND METHODS

Animals

In compliance with NIH and institutional guidelines, CD-1 mice (Charles River Laboratory, Raleigh, NC) were housed in the animal care facility at the University of Kansas Medical Center. Adult female mice (20–25 g, 48–60 days old) were mated with fertile males of the same strain. The morning a vaginal plug was found was designated Day 1 of pregnancy.

Antibody Preparation

The antibodies against CB1-R were raised in rabbits using a synthetic 17-amino acid peptide (81–97, EFYKSLSSFKENEENI) representing the N-terminal region of the rat CB1-R sequence [8]. It should be noted that there is no sequence overlap between the 17-amino acid peptide used as immunogen for raising CB1-R antibodies and any sequence within CB2-R. In brief, rabbits were immunized with the peptide coupled to a carrier protein and bled 10 wk later to collect antisera. The antipeptide antisera were affinity-purified using Affi-gel 10 (Bio-Rad, Richmond, VA) conjugated with the corresponding peptide [19] and used for Western blotting and immunostaining.

Western Blotting

To characterize the specificity of the antibodies, Western blotting was performed with brain membranes or Day 4 blastocysts. Brain membranes were prepared according to the procedure described previously [19]. Rat or mouse brains were homogenized in buffer A (10 mM Tris-HCl [pH 7.4], 250 mM sucrose, 2 mM EGTA, 10 μg/ml leupeptin, 20 μg/ml PMSE, and 10 μg/ml aprotinin), and centrifuged at 800 × g for 10 min at 4°C. The supernatants were re-centrifuged for 1 h at 110 000 × g at 4°C. The pellets were then fixed in 2% formalin in PBS for 15 min and cytospun onto poly-L-lysine-coated glass slides. Embryos were incubated in blocking solution (10% normal goat serum) for 10 min before incubation in affinity-purified rabbit antipeptide antibodies to CB1-R (1 μg/ml) overnight at 4°C. Immunostaining was performed using a Zymed-Histostain-SP Kit for rabbit primary antibody (Zymed Laboratories, San Francisco, CA) [19]. The embryos were counterstained lightly with hematoxylin and mounted.

Culture of Preimplantation Embryos

To study the effects of cannabinoid agonists and/or the CB1-R antagonist on preimplantation embryo development, 2-cell embryos were recovered on Day 2 (0830–0900 h) and pooled from several mice in Whitten’s medium containing 0.3% BSA [20, 21]. They were washed four times
in the same medium before culture. Embryos were cultured in groups of 10 in 25 µl of Whitten's medium under silicon oil in an atmosphere of 5% CO2:95% air at 37°C for 72 h in the absence or presence of various cannabinoid agonists with or without SR141716A, the CB1-R antagonist [15]. The cannabinoid agonists used were CP 55,940 (Pfizer Diagnostics, Groton, CT), WIN 55212–2, and anandamide (Research Biochemicals, Natick, MA). CP 55,940 is a synthetic THC analogue, while WIN 55212–2 is a synthetic noncannabinoid aminoalkylindole that binds with cannabinoid receptors [22]. SR141716A was generously provided by Sanofi Recherche (Montpellier, France). All test agents were dissolved in ethanol and diluted with Whitten's medium. The final ethanol concentration was less than 0.1%. The control cultures contained the same concentration of ethanol. Cannabinoid agonists were added at the beginning of cultures. To examine the reversibility of cannabinoid-induced arrest of embryo development by the CB1-R antagonist, embryos were precultured with SR141716A 1 h before culture with agonists and SR141716A. The concentrations of agonists used were based on our previous results [15]. 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 into blastocysts were subjected to differential cell counting of their inner cell mass and trophectoderm cells [23]. Experiments were repeated 3–6 times, and statistical analysis was performed using chi-square and Fisher exact tests.

RESULTS

Immunoreactive CB1-R Protein in Preimplantation Embryos

Western blotting and immunohistochemistry were used to determine whether the CB1-R mRNA is translated in preimplantation mouse embryos. To determine that the antibodies were specific to CB1-R, Western blotting was performed first. Since the antipeptide antibody was based on the rat CB1-R amino acid sequence, both mouse and rat brain membranes were used for immunoblot analysis. The population of CB1-R is thought to be in higher abundance in the mammalian brain. Two major bands of ~59 kDa and ~54 kDa were detected in rat brain membranes, while a predominant band of ~54 kDa was observed in mouse brain membranes (Fig. 1). In blastocyst preparations, a major band of ~59 kDa and a minor band of ~54 kDa were detected. The ~54-kDa band is consistent with the predicted size of the CB1-R [8]. The ~59-kDa band in the rat brain or mouse blastocyst suggests possible glycosylation of the receptor protein [24]. These bands were not detected when preneutralized antibodies with an excess of the antigenic peptide were used. Three bands of 64-, 59-, and 53-kDa proteins were reported previously in the rat brain [24]. In addition, a ~55-kDa protein was detected in S9 cells expressing the rat CB1-R cDNA from a baculovirus expression vector [25].

Immunohistochemistry was used to detect distribution of CB1-R protein in preimplantation embryos. Little or no immunoreactive CB1-R was detected in 1-cell embryos, while distinct signals were evident in embryos from 2-cell through blastocyst stages. In the morula, immunoreactive CB1-R was detected primarily in outside cells, whereas in the blastocyst, it was detected predominantly in trophectoderm cells. Little or no reactivity was detected in the inner cell mass (Fig. 2). Prenutralized antibodies with an excess of the antigenic peptide failed to show any positive immunostaining in preimplantation embryos (data not shown). The patterns of immunostaining are consistent with our previous observation of autoradiographic distribution of anandamide binding sites in preimplantation mouse embryos [15].

Analysis of Cannabinoid Binding Kinetics in the Blastocyst

Our initial studies showed that all of the 3H-anandamide that could be recovered after incubation with blastocysts for 2 h remains intact. This suggested little or no breakdown of anandamide by blastocysts under these experimental conditions. However, brain membranes were prepared in the buffer containing PMSF as described above for Western blotting, and binding analysis was performed as described [16]. Addition of PMSF has been shown to enhance anandamide binding to brain membranes by preventing its hydrolysis by amidase [26].

Scatchard analysis showed that anandamide binds to a single class of high-affinity sites in Day 4 blastocysts with an apparent $K_d$ of 1.0 nM and $B_{max}$ of 0.09 fmol/blastocyst (Fig. 3A). In contrast, the apparent $K_d$ and $B_{max}$ for Day 4 pregnant mouse brain membranes were 1.8 nM and 18.8 pmol/mg protein, respectively (data not shown). Cannabinoid agonists Win 55212-2 (K; 2.1 nM), CP 55940 (K; 1.8 nM), and (-) THC (K; 1.8 nM), and the CB1-R antagonist SR141716A (K; 1.4 nM), but not the inactive (+) THC (K; 4.8 µM), competed for 3H-anandamide binding in Day 4 blastocysts (Fig. 3B). The apparent $K_d$ (1.2 nM) and $B_{max}$ (0.09 fmol/blastocyst) for WIN 55212–2 binding to blastocysts were also comparable to those of anandamide binding.

Reversal of Cannabinoid-Induced Inhibition of Embryo Development by CB1-R Antagonist

Under our culture conditions, about 90% of 2-cell embryos developed into blastocysts within 72 h in the absence of any cannabinoid agonists. In contrast, when 2-cell em-
bryos were cultured in the presence of anandamide (7 nM), Win 55212–2 (7 nM), or CP 55,940 (4 nM), only 36.4%, 41.9%, or 41.1% of embryos, respectively, developed into blastocysts. Most of the developmental arrest occurred at the 8-cell/morula stage. The adverse effects of cannabinoids on embryo development were reversed when 2-cell embryos were cultured in the presence of SR141716A (8 nM) plus the same concentrations as above of anandamide, WIN 55212–2, or CP 55,940: 87.5%, 87.1%, or 96.5% of 2-cell embryos, respectively, developed into blastocysts. SR141716A (8 nM) alone had no deleterious effects on embryonic development: more than 90% of 2-cell embryos developed into blastocysts (Fig. 4). Embryos that developed into blastocysts in the presence of an agonist plus SR141716A or SR141716A alone were viable, since about 50% of these blastocysts implanted when transferred into uteri of synchronized Day 4 pseudopregnant mice (data not shown).

The adverse effects of cannabinoid agonists were also noted in trophectoderm cell number of blastocysts that escaped the developmental arrest. The CB1-R antagonist SR141716A was also effective in reversing this adverse

**FIG. 2.** Immunohistochemistry of CB1-R in preimplantation mouse embryos. Bright-field microphotographs (×400) of representative (a) 2-cell, (b) 4-cell, (c) morula, and (d) blastocyst are shown. Reddish-brown deposits indicate positive immunostaining. Incubation of embryos in antibodies preneutralized with excess of antigenic peptide showed no positive staining (data not shown). tr, trophectoderm; icm, inner cell mass.

**FIG. 3.** Characterization of cannabinoid binding in Day 4 blastocysts. A) Scatchard analysis of 3H-anandamide binding in Day 4 mouse blastocysts. Inset: equilibrium binding kinetics. The apparent \( K_d \) and \( B_{max} \) of anandamide binding in the embryo were 1.0 nM and 0.09 fmol/blastocyst, respectively, while those in the brain were 1.8 nM and 18.8 pmol/mg membrane protein, respectively (data not shown). B) Competition by cannabinoid agonists CP 55,940, WIN 55212–2, (−)THC (active cannabinoid), (+)THC (inactive cannabinoid), or SR141716A (CB1-R antagonist) for anandamide binding in Day 4 blastocysts. While CP 55,940 (Kd 1.8 nM), WIN 55212–2 (Kd 2.1 nM), (−)THC (Kd 1.8 nM), or SR141716A (Kd 1.4 nM) efficiently displaced anandamide binding, (+)THC (Kd 4.8 μM) was a poor competitor.
Day 4 mouse blastocysts [27, 28], it is apparent that mouse receptor subtype is functional in preimplantation mouse embryos. The results of Western blotting also suggest that the CB2-R has a large population of high-affinity receptors in the embryo. Nominal concentrations are consistent with the presence of cannabinoid binding sites in the blastocyst, and, considering the typical number (-50) and protein content (20 ng) of mouse embryo. The inhibitory effects of cannabinoid agonists on preimplantation embryo development at low nanomolar concentrations are consistent with the presence of the brain-type receptors. CP 55,940, (-)THC, and anandamide have each suggested N-linked glycosylation of the rat brain CB1-R [17, 18]. The successful competition of cannabinoid agonists with SR141716A. The number of inner cell mass cells was not significantly altered under any of these experimental conditions.

**DISCUSSION**

The highlights of the investigation presented here are that the CB1-R mRNA undergoes translation and that this receptor subtype is functional in preimplantation mouse embryos. Scatchard analysis of [3H]-anandamide or [3H]-WIN 55212–2 suggests a high-affinity single class of cannabinoid binding sites in the blastocyst, and, considering the total cell number (~50) and protein content (~20 ng) of Day 4 mouse blastocysts [27, 28], it is apparent that mouse blastocysts have many more high-affinity cannabinoid receptors than does the mouse brain. This is perhaps the first report of any Scatchard analysis in the preimplantation mouse embryo. The inhibitory effects of cannabinoid agonists on preimplantation embryo development at low nanomolar concentrations are consistent with the presence of a large population of high-affinity receptors in the embryo. The results of Western blotting also suggest that the CB1-R protein is in higher abundance than that in the brain. Rat brain CB1-R has consensus sequence for three potential sites of N-linked glycosylation on the extracellular N-terminus of the receptor [8], and Western blot analysis has confirmed N-linked glycosylation of the rat brain CB1-R [24]. Our results suggest that the mouse blastocyst CB1-R, like the rat brain CB1-R, is primarily glycosylated, in contrast to the mouse brain CB1-R. However, confirmation of this observation using glycosidase treatments could not be accomplished at this time because of the requirement for a large number of blastocysts.

The reversal of the cannabinoid-induced inhibition of embryonic development by a CB1-R antagonist (SR-141716A) at a low nanomolar concentration strongly suggests that the brain-type receptors are primarily responsible for the observed effects of cannabinoids. The primary effects of cannabinoids appear to be targeted at the outer cells of embryos that constitute the trophectoderm. This is consistent with the patterns of cannabinoid binding [15] and immunostaining and with the reduction of trophectoderm cells in blastocysts.

Although the CB2-R mRNA is expressed [15], it is not yet known whether this receptor mRNA is efficiently translated in the preimplantation mouse embryo or whether this receptor subtype has any functions in the preimplantation embryo. SR141716A is considered to be a preferred antagonist to CB1-R. This compound displays 1000-fold higher affinity for the central receptors (CB1-R) than for peripheral receptors (CB2-R) [17, 18]. The successful competition of anandamide binding by SR141716A with a Kᵢ of 1.4 nM suggests that cannabinoid binding sites are primarily in the brain type. CP 55,940, (-)THC, and anandamide have each been observed to be essentially equipotent in interacting with CB1-R and with CB2-R, although WIN 55212–2 and cannabinoil were each found to bind to CB2-R with higher affinity than to the CB1-R [29]. The availability of specific antibodies, or a specific antagonist or agonist to CB2-R will be required to explore the interactions of cannabinoid agonists with this receptor subtype and its roles in preimplantation embryos.

Cannabinoid receptors are coupled to pertussis toxin-sensitive Gi, and activation of these receptors interferes with adenylyl cyclase and calcium (Ca2⁺) currents [7, 8]. These two signal-transducing second messengers are implicated in cell proliferation, differentiation, and gene expression. Indeed, cAMP is associated with embryonic gene activation and blastocyst expansion, while intracellular Ca2⁺ is implicated in cell polarity and embryonic compaction necessary for morula-to-blastocyst transformation (reviewed in [15]). The failure of embryos to proceed beyond the 8-cell stage and reduced trophectoderm cell numbers in

**TABLE 1. Effects of cannabinoid agonists and/or CB1-R antagonist on trophectoderm (Tr) and inner cell mass cell (ICM) number of blastocysts (mean ± SEM).**

<table>
<thead>
<tr>
<th>Treatments</th>
<th>No. of Tr cells</th>
<th>No. of ICM cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (&lt; 0.1% ethanol)</td>
<td>44.2 ± 1.2 (27)</td>
<td>10.3 ± 0.3 (25)</td>
</tr>
<tr>
<td>SR141716A</td>
<td>44.7 ± 1.1 (19)</td>
<td>10.7 ± 0.4 (17)</td>
</tr>
<tr>
<td>Anandamide</td>
<td>28.4 ± 1.6 (5)</td>
<td>8.0 ± 1.0 (5)</td>
</tr>
<tr>
<td>SR141716A + anandamide</td>
<td>45.6 ± 1.0 (8)</td>
<td>10.0 ± 0.4 (6)</td>
</tr>
<tr>
<td>WIN 55212-2</td>
<td>35.8 ± 1.7 (6)</td>
<td>9.5 ± 0.2 (6)</td>
</tr>
<tr>
<td>SR141716A + WIN 55212-2</td>
<td>45.4 ± 1.4 (11)</td>
<td>11.1 ± 0.4 (11)</td>
</tr>
<tr>
<td>CP 55,940</td>
<td>34.3 ± 1.6 (13)</td>
<td>10.3 ± 0.6 (13)</td>
</tr>
<tr>
<td>SR141716A + CP 55,940</td>
<td>44.8 ± 1.3 (14)</td>
<td>10.2 ± 0.4 (14)</td>
</tr>
</tbody>
</table>

*The numbers in parentheses indicate the number of blastocysts analyzed in each group.*
These results suggest that inhibitory effects of cannabinoids, recently observed that continuous s.c. infusion of active ligands or by some other mechanisms. However, we have effects of cannabinoids are mediated directly via these receptors [31-33], it is still not known whether the in vivo findings of retarded embryonic development and pregnancy failure after chronic exposure to exogenous cannabinoids [31-33], is it not still known whether the in vivo effects of cannabinoids are mediated directly via these receptors or by some other mechanisms. However, we have recently observed that continuous s.c. infusion of active cannabinoids from Day 2 of pregnancy in the mouse interferes with implantation when examined on Day 5, and that this inhibition is reversed by simultaneous infusion of the CB1-R antagonist SR141716A (our unpublished results). These results suggest that inhibitory effects of cannabinoids on implantation are also mediated by CB-1 receptors. Collectively, our findings establish the preimplantation embryo as a target for cannabinoid ligand-receptor signaling that involves primarily brain-type cannabinoid receptor.

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