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Role of the endocannabinoid system in the control of mouse myometrium contractility during the menstrual cycle

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ABSTRACT

Cannabis and cannabinoids are known to affect female reproduction. However, the role of the endocannabinoid system in mouse uterine contractility in the dioestrus and oestrus phases has not been previously investigated. The present study aimed at filling this gap. Endocannabinoid (anandamide and 2-arachidonoylglycerol) levels were measured in mouse uterus at dioestrus and oestrus phases by liquid chromatography-mass spectrometry; quantitative reverse transcription-PCR and western blot were used to measured the expression of cannabinoid receptors and enzymes involved in the metabolism of endocannabinoids. Contractility was evaluated *in vitro* either on the spontaneous contractions or by stimulating the isolated uterus with exogenous spasmogens. The tissue concentrations of anandamide and 2-AG were reduced in the oestrus phase, compared to dioestrus. Uteri obtained in the dioestrus, but not oestrus, phase showed spontaneous phasic prostaglandin-mediated contractions that were reduced by ACEA (CB_1 receptor agonist) and to a lower extent by JWH133 (CB_2 receptor agonist). These inhibitory effects were counteracted by the corresponding selective antagonists. Neither ACEA nor JWH133 did affect the contractions induced by exogenous PGE_2 in the uterus from the oestrus phase. The FAAH inhibitor JNJ1661010 and, to a lower extent, the MAGL inhibitor JZL184 also reduced spontaneous contractions. It is concluded that the endocannabinoid system undergoes to adaptive changes between the oestrus and dioestrus phases. CB_1 and, to a lower extent, CB_2 receptor activation results in selective inhibition of myometrial contractility, without un-specific relaxing effects on the smooth muscle. These results might be of interest for female marijuana smokers as well as for the design of novel tocolytic agents.

1. Introduction

Marijuana, a preparation from the *Cannabis sativa* plant, continues to be the most frequently used illicit drug among women of childbearing age [1] and its use has been demonstrated to affect adversely reproduction [2]. Women smoking marijuana show impaired fertility, aberrant hormonal regulation, or impaired embryo implantation and development [3]. Δ^9-tetrahydrocannabinol (Δ^9-THC), the main psychotropic component of marijuana, binds two $G_{i/o}$ coupled membrane receptors, named CB_1 and CB_2 receptors, which are also activated by endogenous ligands (endocannabinoids). The main endocannabinoids are anandamide and 2-arachydonoylglycerol (2-AG), and are generated on demand rather than stored in cells. Endocannabinoids are biosynthesized from membrane phospholipids by the action of a number of enzymes including *N*-acyl-phosphatidylethanola mine-selective phospholipase D (NAPE-PLD, involved in

Abbreviations: AEA, anandamide; 2-AG, 2-arachydonoylglycerol; DAGL, diacylglycerol lipases; ECS, endogenous cannabinoid system; FAAH, fatty acid amide hydrolase; MAGL, monoacylglycerol lipase; NAPE-PLD, *N*-acyl-phosphatidy lethanolamine-selective phospholipase D; OEA, oleoylethanolamide; PEA, palmitoylethanolamide; Δ^9-THC, Δ^9-tetrahydrocannabinol.

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anandamide biosynthesis) and diacylglycerol lipases α and β (DAGL α and β, involved in 2-AG biosynthesis), and are inactivated through a reuptake process (facilitated by a putative endocannabinoid membrane transporter), followed by enzymatic degradation. The latter process occurs predominantly through fatty acid amide hydrolase (FAAH, in the case of anandamide) and through the serine hydrolases monoacylglycerol lipase (MAGL), and α,β-hydrolase 6 (ABHD6) and 12 (in the case of 2-AG) [4].

The endocannabinoid system has been found in the female reproductive system of different species, from sea urchins to humans, thus suggesting its possible role in female reproduction [5]. Components of the endocannabinoid system have been identified in the rodent and human uterus and changes in anandamide synthesis and/or expression of cannabinoid receptors in this organ have been suggested to be responsible for early pregnancy failure or female infertility [6]. In addition, endogenous and exogenous cannabinoid receptor agonists, including THC and anandamide, exert a direct, CB_1-mediated relaxant effect on myometrial contractility *in vitro* [7]. Despite these reports, no conclusive data exist on a possible tonic action of the endocannabinoid system on the contractility of the myometrium. Specifically, the possible involvement of the endocannabinoid system in the oestrus phase (i.e. when the uterus is ready for implantation) and dioestrus phase (i.e. when ovulation normally occurs) is not known to date. In the present study, first we have measured endocannabinoid levels as well the mRNA and protein expression of cannabinoid receptors and enzymes involved in endocannabinoids biosynthesis and degradation in the uterus from mice in dioestrus and oestrus phases. Then, we have investigated the role of the endocannabinoid system in uterine smooth muscle contractility in both phases. For this latter purpose, we used the selective cannabinoid CB_1 receptor agonist, ACEA; the selective CB_2 receptor agonist, JWH133; the selective CB_1 receptor and CB_2 receptor antagonists, rimonabant and SR144528; the selective FAAH inhibitor, JNJ16610, the selective MAGL inhibitor, JZL184 and the ABDH6 inhibitor, WWWL70 [8].

2. Material and methods

2.1. Chemicals

Prostaglandin E_2, prostaglandin $F_{2\alpha}$, acetylcholine chloride (ACh), atropine sulphate, ω-conotoxin, verapamil hydrochloride, cyclopiazonic acid, indomethacin, tetrodotoxin (TTX), SC19220, L-798,106 and glibenclamide were purchased from Sigma (Milan, Italy). ACEA, JWH133, JNJ1661010, JZL184, WWL70 and SQ22536 were purchased from Tocris (Bristol, UK). AL 8810 was obtained from Cayman Chemical (Cabru SAS, Arcore, Italy). SR 141716 (or rimonabant) 5-(4-Chlorophenyl)-1-(2,4-dichloro-phenyl)-4-methyl-N-(piperidin-1-yl)-1H-pyrazole-3-carboxamide, SR144528 (N-[-1S-*endo*-1,3,3-trimethyl bicyclo heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide), SR140333 (S)1-{2-[3-(3,4-dichlorophenyl)-1-(3-isopropoxypheny lacetyl)piperidin-3-yl-]ethyl}-4-phenyl-1-axoniabicyclo octane chloride, SR 48968 (S)-N-methyl-N[4-(4-acetylamono-4-phenylpi peridino)-2-(3,4-dichlorophenyl)-butyl]benzamide hydrochloride and SR 142801 (S)-(N)-(1–3-(1-ben-zoyl-3-(3,4-dichlorophenil)pi peridin-3-yl)propyl)-4-phenylpiperi-din-4-yl)-N-methylacetamide were a kind gift from Sanofi-Aventis (Montpellier, France).

ACh, atropine, tetrodotoxin, verapamil, ω-conotoxin and SR 48968 were dissolved in distilled water. Cyclopiazonic acid, indomethacin, SR142801, SC 19220, L-798,106, AL 8810, JWH133, JNJ1661010, JZL184, rimonabant, SR144528, WWL70, glibenclamide and SQ22536 were dissolved in dimethyl sulfoxide (DMSO) and SR 140333 in DMSO/water (50%, v/v). ACEA, prostaglandin E_2 and prostaglandin F2α were dissolved in ethanol. The drug vehicles had no significant effect on the responses under study.

2.2. Animals

Sexually mature virgin female ICR mice (22–28 g) purchased from Charles River Italia (Lecco, Italy) were housed in polycarbonate cages in isolators under a 12-h light/12-h dark cycle, temperature 23 ± 2° C and humidity 60%. Animals, used after 1 week of acclimation, had free access to water and food. Mice were used in oestrus and dioestrus phase thus to obtain uteri from mice in dioestrus phase (dioestrus tissues) and uteri from mice in oestrus phase (oestrus tissues). Stage of dioestrus or oestrus cycle was determined by examination of fresh vaginal smears as previously described [9]. All experiments complied with the Italian D.L. no. 116 of 27 January 1992 and associated guidelines in the European communities Council (86/609/ECC and 2010/63/UE).

2.3. Western blot analysis

Female mice (in oestrus and dioestrus phases) were killed by asphyxiation with carbon dioxide and whole uteri were collected carefully to avoid excessive stretching, cleaned from fat tissue, and immediately frozen in liquid nitrogen. For sample preparation, each tissue was washed twice in cold PBS (without Ca2+ and Mg2+, pH 7.4) and homogenized in 0.5 ml of TNE lysis solution (EDTA 0.5 M; NaCl (5 M); Tris-Cl (1 M, pH 7.5); 0.1% Triton plus protease inhibitors protease inhibitors). Lysates were then placed on an orbital shaker and incubated at 4 °C for 30 min. Lysates were then centrifuged for 15 min at 13,000*g* at 4° C, and the supernatants transferred into clear tubes and quantified by DC Protein Assay (Bio-Rad, Segrate, MI, Italy). Subsequently, the samples (70 μg of total protein) were incubated for 10 min in bolt buffer plus sample reducing agents (Thermo Fisher Scientific, Milan Italy) and loaded on bis-tris plus precast polyacrylamide gel (4–12%, Thermo Fisher Scientific, Milan Italy) and then transferred to a PVDF membrane. Filters were incubated overnight at 4 °C with the following antibodies: (a) policlonal anti-CB_1 (cat. No. Y080037; Applied Biological Materials Inc.; Canada); (b) monoclonal anti-CB_2 (cat. No. WH0001269M1; Sigma-Aldrich, Milan Italy); (c) mouse anti-FAAH (cat. N. WH0002166M7; Sigma–Aldrich, Milan Italy); (d) policlonal anti-NAPE-PLD (Cat. No. ABIN2786441; Abnova, Taiwan); (e) anti-MAGL (Cat. No. 100035; Cayman, USA); (f). The monoclonal anti-tubulin (Cat. No. T8203; Sigma–Aldrich) was used to check for equal protein loading. Reactive bands were detected by chemiluminescence (ECL or ECL-plus; Perkin-Elmer). Images were analysed on a Chemi-Doc station with Quantity-one software (Bio-Rad, Segrate, MI, Italy) [10].

2.4. Reverse transcription polymerase chain reaction (RT-PCR) analysis

All uterine tissues were collected immediately after sacrifice and immersed in RNA later (Invitrogen, Carlsbad, CA, USA). Tissue samples were homogenized by a rotorstator in Trizol (Invitrogen) and total RNA was purified, quantified, characterized and *retro*-transcribed as previously described [11]. Total RNA was purified, quantified, characterized and *retro*-transcribed as previously described [11]. For all samples tested, the RNA integrity number (Bionalyzer 2100, Agilent) was greater than 8 relative to a 0–10 scale. Quantitative real-time PCR was performed by an iCycler-iQ5® in a 20 μL reaction mixture as described. Assays were performed in quadruplicate (maximum ΔCt of replicate samples <0.5), and a standard curve from consecutive fivefold dilutions (100–0.16 ng) of a cDNA pool representative of all samples was included for PCR efficiency determination. Optimized primers for