Molecular Human Reproduction Vol.10, No.4 pp. 215–221, 2004 DOI: 10.1093/molehr/gah034

Mouse blastocysts release a lipid which activates anandamide hydrolase in intact uterus

M.Maccarrone[1,4], M.DeFelici[2], F.G.Klinger[2], N.Battista[3], F.Fezza[1], E.Dainese[1], G.Siracusa[2] and A.Finazzi-Agrò[3]

[1]Department of Biomedical Sciences, University of Teramo, Piazza A. Moro 45, Teramo 64100, Italy, and Departments of [2]Public Health and Cell Biology and [3]Experimental Medicine and Biochemical Sciences, University of Rome 'Tor Vergata', Rome 00133, Italy

[4]To whom correspondence should be addressed. E-mail: Maccarrone@vet.unite.it

Anandamide (*N*-arachidonoylethanolamine, AEA) is a major endocannabinoid, known to impair mouse pregnancy and embryo development and to induce apoptosis in blastocysts. Here we show that mouse blastocysts rapidly (within 30 min of culture) release a soluble compound, that increases by ~2.5-fold the activity of AEA hydrolase (fatty acid amide hydrolase, FAAH) present in the mouse uterus, without affecting *FAAH* gene expression at the translational level. This 'FAAH activator' was produced by both trophoblast and inner cell mass cells, and its initial biochemical characterization showed that it was fully neutralized by adding lipase to the blastocyst-conditioned medium (BCM), and was potentiated by adding trypsin to BCM. Other proteases, phospholipases A_2, C or D, DNAse I or RNAse A were ineffective. BCM did not affect the AEA-synthesizing phospholipase D, the AEA-binding cannabinoid receptors, or the selective AEA membrane transporter in mouse uterus. The FAAH activator was absent in uterine fluid from pregnant mice and could not be identified with any factor known to be released by blastocysts. In fact, platelet-activating factor inhibited non-competitively FAAH in mouse uterus extracts, but not in intact uterine horns, whereas leukotriene B_4 or prostaglandins E_2 and $F_2\alpha$ had no effect. Overall, it can be suggested that blastocysts may protect themselves against the noxious effects of uterine endocannabinoids by locally releasing a lipid able to cross the cell membranes and to activate FAAH. The precise molecular identity of this activator, the first ever reported for FAAH, remains to be elucidated.

Key words: anandamide/enzyme activation/fertility/hydrolysis/implantation

Introduction

Endocannabinoids are amides, esters and ethers of long chain polyunsaturated fatty acids, found in several human tissues (Fowler *et al*., 2001; Hanus *et al*., 2001), and in human reproductive fluids (Schuel *et al*., 2002). Anandamide (*N*-arachidonoylethanolamine, AEA) and 2-arachidonoylglycerol are the main endocannabinoids described to date (Howlett and Mukhopadhyay, 2000). They bind to both type-1 (CB1) and type-2 (CB2) cannabinoid receptors, thus mimicking several central and peripheral effects of Δ^9-tetrahydrocannabinol (THC), the main active ingredient of hashish and marijuana (Mechoulam and Hanus, 2000). At the periphery, AEA and 2-arachidonoylglycerol show cardiovascular (Kunos *et al*., 2000), immune (Parolaro *et al*., 2002) and anti-inflammatory activities (De Petrocellis *et al*., 2000). Moreover, endogenous cannabinoids have been involved in the inhibition of human breast and prostate cancer cell proliferation (Melck *et al*., 2000). The effect of AEA via CB1 and CB2 receptors depends on its extracellular concentration, which is controlled by (i) cellular uptake by a specific AEA membrane transporter (AMT), and (ii) intracellular degradation by the AEA-hydrolysing enzyme fatty acid amide hydrolase (FAAH). AMT (Hillard and Jarrahian, 2000) and FAAH (Ueda *et al*., 2000) have been characterized in several mammalian cells and tissues, and together with AEA and congeners form the 'endocannabinoid system'.

Evidence is accumulating about endocannabinoid modulation of embryo–uterine interactions (Paria and Dey, 2000), and impairment of pregnancy and embryo development in mice (Yang *et al*., 1996), thus recalling the adverse effects of THC on reproduction (Hall and Solowij, 1998). More recently, progesterone has been shown to be involved in THC modulation of sexual receptivity in female rats (Mani *et al*., 2001), and dysregulation of cannabinoid signalling has been shown to disrupt uterus receptivity to embryo implantation in mice (Paria *et al*., 2001). Along this line, we reported the association between decreased FAAH activity and expression in maternal peripheral lymphocytes and early pregnancy failure in humans, demonstrating that an impairment of AEA degradation might be connected with reduced fertility (Maccarrone *et al*., 2000a). Consistently, down-regulation of AEA levels in mouse uterus has been associated with increased uterine receptivity, which instead decreases when AEA is up-regulated (Yang *et al*., 1996; Schmid *et al*., 1997). Mouse uterus contains the highest levels of AEA so far detected in mammalian tissues, and is the only tissue where AEA appears to be the main (up to 95%) *N*-acylethanolamine (Schmid *et al*., 1997). Furthermore, the developmental arrest that occurs in mouse blastocysts in a non-receptive uterine environment correlates well with its higher levels of AEA and with the *in vitro* observation that AEA inhibits embryo development and zona-hatching of blastocysts (Paria *et al*., 1996; Yang *et al*., 1996; Schmid *et al*., 1997).

Mouse blastocysts express a biologically active CB1 receptor, together with specific AMT and FAAH (Maccarrone *et al*., 2000b). They are driven to apoptosis by AEA, suggesting that an efficient

degradation of this compound might be instrumental in preventing its blastotoxic effects (Maccarrone *et al*., 2000b). As a matter of fact, recent observations on FAAH mRNA accumulation (Paria *et al*., 1999) and FAAH activity (Maccarrone *et al*., 2000b) in pregnant mice suggest that this enzyme may be the 'checkpoint' for maintaining the steady levels of AEA. Indeed, FAAH activity has recently been shown to correlate inversely with AEA levels in various tissues of FAAH knockout mice versus wild-type animals (Cravatt *et al*., 2001) and in blood of women with normal gestation versus those who aborted (Maccarrone *et al*., 2002). Here, we report that blastocysts release a soluble factor able to activate uterine FAAH, thus reducing the level of AEA and its negative effects on blastocyst survival and implantation.

Materials and methods

Materials and enzymes

All chemicals were of the purest analytical grade. Leukotriene B_4 (LTB_4) and prostaglandins E_2 (PGE_2) and $F_2\alpha$ ($PGF_2\alpha$) were from Cayman Chemical Company (USA). Platelet activating factor-16 (1-*O*-palmityl-2-acetyl-*sn*-glycero-3-phosphocholine; PAF-16) and -18 (1-*O*-stearyl-2-acetyl-*sn*-glycero-3-phosphocholine; PAF-18), deoxyribonuclease I (from bovine pancreas; DNAse I), ribonuclease A (from bovine pancreas; RNAse A), phospholipases C (from *Bacillus cereus*) and D (from *Streptomyces chromofuscus*), and lipase (from *Chromobacterium viscosum*) were from Calbiochem (USA). Anandamide (*N*-arachidonoylethanolamine, AEA), cycloheximide, phospholipase A_2 (from *Apis mellifera*), trypsin and α-chymotrypsin (from bovine pancreas), bromelain (from pineapple stem), papain (from *Papaya latex*), and pepsin A (from porcine stomach) were from Sigma Chemical Company (USA). Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum (FCS) were from Life Technologies (Italy). *N*-(4-hydroxyphenyl)Arachidonoylamide (AM404) was from Research Biochemicals International (USA). Methyl-arachidonoyl fluorophosphonate (MAFP) was from Cayman Chemicals (USA). *N*-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole-carboxamide (SR 141716) and *N*-[1(*S*)-endo-1,3,3-trimethyl bicyclo [2.2.1] heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide (SR 144528) were kind gifts from Sanofi Recherche (France). [^{3}H]AEA (223 Ci/mmol) and [^{3}H]CP55.940 (5-(1,1′-dimethyheptyl)-2-[1*R*,5*R*-hydroxy-2*R*-(3-hydroxypropyl) cyclohexyl]-phenol; 126 Ci/mmol) were from NEN DuPont de Nemours (Germany). 1,2-Dioleoyl-3-phosphatidyl[2-^{14}C]ethanolamine (55 mCi/mmol) was from Amersham Pharmacia Biotech (Sweden). Anti-FAAH polyclonal antibodies were raised in rabbits against the conserved FAAH sequence VGYYETDNYTMPSPAMR (Giang and Cravatt, 1997) conjugated to ovalbumin, and were prepared by Primm S.r.l. (Italy). Goat anti-rabbit antibodies conjugated to alkaline phosphatase (GAR-AP) were from Bio-Rad (USA).

Blastocyst culture and collection of conditioned medium

CD-1 mice were purchased from Charles River (Italy). Uterine horns were excised from virgin female mice at a random stage of the estrous cycle as reported (Maccarrone *et al*., 2000b), and early blastocysts (3.5 days *post coitum*) were collected in the mid morning by flushing the uterus with M2 medium plus 1 mg/ml polyvinylpyrrolidone (Sigma) (Maccarrone *et al*., 2000b). Twenty-five blastocysts were then cultured in 20 µl M16 medium in Terasaki's 96 microwells for the indicated periods of time, at 37°C and 5% CO_2 in humidified incubator. At the end of the incubation, blastocysts were removed and the blastocyst-conditioned medium (BCM) was used as such for subsequent analysis. In some experiments, blastocysts were killed by rapid freezing (–80°C) and thawing (37°C) and were then kept at 37°C in M16 medium for 12 h. In other experiments, morulae (2.5 days *post coitum*) or late blastocysts (4.5 days *post coitum*) were used to produce conditioned media (CM) with the same procedure described above (25 morulae or late blastocysts in 20 µl M16 medium for the indicated periods, at 37°C). Morulae and late blastocysts were collected by flushing the oviduct and uterus respectively. In yet other experiments, blastocysts were obtained following *in vitro* culture of 2-cell embryos (2-CE) in 0.5 ml M16 medium plus 4 mg/ml bovine serum albumin (fraction V; Sigma) in a well of a 4-well Nunclon dish (Nunc, Denmark) for 4 days at 37°C in 5% CO_2. At the end of the culture period, 2-CE-derived blastocysts were washed and cultured for 2 h in M16 medium in Terasaki's microwells, in order to prepare CM as described above. With the aim of ascertaining the source of the FAAH activator in the blastocyst, trophoblast and inner cell mass (ICM) cells were separated as follows. Briefly, 25 early blastocysts were cultured in 20 µl M16 + 10% FCS (Life Technologies) for 5 days in Terasaki's microwells at 37°C in 5% CO_2. Under these conditions, trophoblast cells attach and spread onto the culture dish, forming a monolayer, whereas ICM cells remain on top of the trophoblast cells. Using a mouth-operated glass micropipette, ICM cells were displaced from the trophoblast cell monolayer and transferred to another well. CM was prepared by incubating 25 trophoblasts or 25 ICM cells in 20 µl M16 for 2 h as reported above. Finally, the presence of the FAAH activator was analysed in uterine fluids and in STO cell monolayers (mouse embryonic fibroblasts, from American Tissue and Cell Collection, UK). Uterine fluids were obtained by flushing uterine horns of pregnant CD-1 mice at 3.5 days *post coitum* with ~100 µl of M16 per horn. STO cells were grown in DMEM supplemented with 10% FCS under standard culture conditions. CM was prepared by incubating STO cells (~8×10^5 cells/ml) in M16 as described above.

The effect of various enzymes on BCM was assessed by incubating the conditioned medium with each enzyme (10 IU) for 1 h at 37°C, before using BCM in FAAH activity assays (see below). All procedures were approved by the Animal Care Committee of University of Teramo and University of Rome 'Tor Vergata'.

Assay of FAAH activity and expression

Fatty acid amide hydrolase (E.C. 3.5.1.4; FAAH) activity in extracts of mouse uterine horns was determined by reversed-phase high performance liquid chromatography, following the release of [^{3}H]arachidonic acid from 10 µmol/l [^{3}H]AEA at pH 9.0 as reported (Maccarrone *et al*., 1999). Tissues were homogenized by UltraTurrax T25 in 50 mmol/l Tris–HCl and 1 mmol/l EDTA, pH 7.4, at a 1:10 homogenization ratio (fresh weight/volume), then the homogenate was centrifuged sequentially at 800 *g* for 5 min and 11 000 *g* for 20 min at 4°C (Maccarrone *et al*., 1999). The final pellet was resuspended in ice-cold buffer at a protein concentration of 1 mg/ml and stored at –80°C until use. FAAH activity was assayed in uterine extracts in the presence of BCM (or plain medium in the controls), or of various compounds added directly to the assay buffer (100 µl). In the case of PAF-16 and PAF-18, the inhibition constant (K_i) of FAAH-catalysed hydrolysis of [^{3}H]AEA (in the range 0–25 µmol/l) was determined by Lineweaver–Burk analysis of the experimental data (Maccarrone *et al*., 1999). FAAH activity was also assayed in intact uterine horns excised from virginal female mice at a random stage of the estrous cycle. Horns (2/test) were pre-incubated at 37°C for 2 h in phosphate-buffered saline (100 µl), containing BCM (or plain medium in the controls) or other compounds, and were then washed and homogenized in assay buffer for subsequent analysis of FAAH activity and expression. For each animal, one horn was treated with BCM and the other one with plain medium as a control.

The FAAH protein content in uterine horns was assessed by enzyme-linked immunosorbent assay (ELISA), as reported (Maccarrone *et al*., 2001). Wells were coated with tissue homogenates (20 µg of proteins/well) prepared as described above for FAAH assay. Wells were then reacted with anti-FAAH antibodies (diluted 1:300), using GAR-AP (diluted 1:2000) as secondary antibody (Maccarrone *et al*., 2001).

Determination of AMT activity, CBR binding and PLD activity in mouse uterus

The uptake of 400 nmol/l [^{3}H]AEA by the AEA membrane transporter (AMT) of synaptosome-like vesicles prepared from uterine horns was determined as described (Battista *et al*., 2002). To discriminate non-carrier-mediated from carrier-mediated transport of AEA through cell membranes, [^{3}H]AEA uptake at 4°C was subtracted from that at 37°C (Hillard *et al*., 1997).

For cannabinoid receptor studies, membrane fractions were prepared from fresh uterine horns as reported (Maccarrone *et al*., 2000c), and were immediately used in rapid filtration assays with the synthetic cannabinoid [^{3}H]CP55.940 at 400 pmol/l (Maccarrone *et al*., 2000c). Non-specific binding was assessed in the presence of 10 µmol/l AEA (Deutsch *et al*., 1997).

The activity of phospholipase D (E.C. 3.1.4.4; PLD) was assayed in homogenates of uterine horns by measuring the release of [^{14}C]ethanolamine

from 1,2-dioleoyl-3-phosphatidyl-[2-^{14}C]ethanolamine (10 µmol/l), as described (Gubellini *et al*., 2002).

Statistical analysis

Data reported in this paper are the mean (± SD) of at least three independent determinations, each in duplicate. Statistical analysis was performed by the non-parametric Mann–Whitney test, analysing experimental data by means of the InStat 3 program (GraphPAD Software for Science, USA).

Results

BCM activates uterine FAAH

The addition of blastocyst-conditioned medium (BCM), i.e. medium in which early blastocysts had been cultured for 2 h, to extracts of mouse uteri enhanced FAAH activity in a dose-dependent manner, while plain medium had no effect (Figure 1A and data not shown). In addition, BCM (up to 10 µl) did not show any ability to hydrolyse AEA, ruling out that it might contain FAAH activity. A dose-dependent, yet less pronounced, activation of FAAH was also observed when intact uterine horns were preincubated for 2 h in BCM, and then assayed for FAAH activity under the same experimental conditions (Figure 1A). In both cases, FAAH activation reached statistical significance ($P < 0.05$) at 2 µl BCM and a maximum at 4 µl BCM. Therefore, the latter amount was chosen to further investigate the effect of BCM on FAAH.

The ability of BCM to activate FAAH, both in uterine homogenates and intact uterine horns, depended on the duration of the preincubation of blastocysts in culture medium, reaching statistical significance ($P < 0.05$) at 30 min and a maximum at 2 h, with no further increase in the following 10 h (Figure 1B). Therefore, BCM in which blastocysts had been cultured for 2 h was used in all further analyses, unless stated otherwise. The medium in which dead blastocysts had been kept for 12 h was unable to enhance FAAH activity (Figure 1B). CM obtained from morulae or late blastocysts enhanced FAAH activity in uterine extracts up to 150 and 330% of the controls, compared to the 250% increase induced by BCM (shown in Figure 1A). The difference between the effect of BCM and that of the medium conditioned with morulae or with late blastocysts was statistically significant ($P < 0.05$ in both cases). CM from blastocysts derived from 2-CE showed a similar ability to enhance FAAH in uterine extracts compared to CM from freshly collected blastocysts (200 versus 250% over the untreated controls respectively).

Western blot analyses of extracts of mouse uterus had shown that specific anti-FAAH antibodies recognized a single immunoreactive band of the molecular size expected for FAAH (Maccarrone *et al*., 2000b). Anti-FAAH antibodies were used here to quantify FAAH content by ELISA, which showed that enhancement of FAAH activity by BCM was not paralleled by an increase of FAAH protein (Table I). Accordingly, enzyme activity was almost abolished by 100 nmol/l MAFP, a selective and powerful irreversible inhibitor of FAAH (Gubellini *et al*., 2002), in both BCM-treated and control mouse uterus

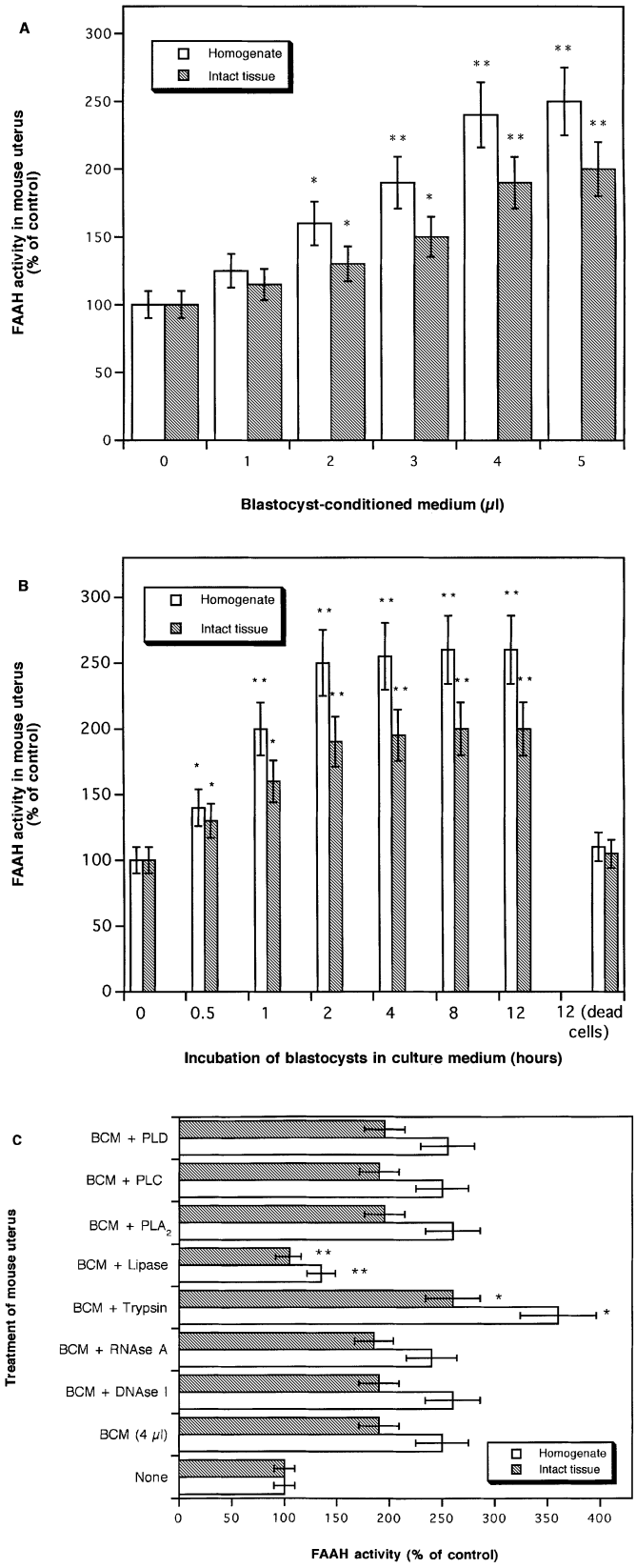

Figure 1. Effect of blastocyst-conditioned medium (BCM) on fatty acid amide hydrolase (FAAH) activity in mouse uterus. (**A**) FAAH activity in uterine tissue homogenates that had been exposed to various volumes of blastocyst-conditioned medium (25 blastocysts/20 µl per test, 37°C for 2 h) before or after homogenization, and referred to as intact tissue or homogenate respectively. (**B**) Dependence of FAAH activity in the same uterine tissue homogenates on the duration of the pre-incubation of blastocysts in culture medium (25 blastocysts/20 µl per test). In each assay, 4 µl BCM was used. (**C**) Effect of various enzymes (10 IU each) on the ability of 4 µl BCM in which blastocysts had been cultured for 2 h to enhance FAAH activity in tissue homogenates or in intact horns. The proteases α-chymotrypsin, bromelain, papain and pepsin A (10 IU each) had no effect, and were omitted for the sake of clarity. In all panels, 100% = 170 ± 18 pmol/min/mg of protein. (**A**, **B**) *$P < 0.05$, **$P < 0.01$ versus controls ($P > 0.05$ in all other cases). (**C**) *$P < 0.05$, **$P < 0.01$ versus BCM-treated samples ($P > 0.05$ in all other cases).

Table I. Effect of a 2 h preincubation of intact mouse uterus with blastocyst-conditioned medium (BCM) on fatty acid amide hydrolase (FAAH) activity and protein

Compound added	FAAH (%)[a]	
	Activity	Protein content
None	100	100
BCM (4 µl/100 µl)	190 ± 20*	100 ± 10
MAFP (100 nmol/l)	20 ± 4*	90 ± 10
BCM (4 µl) + MAFP (100 nmol/l)	35 ± 5*	100 ± 10
Cycloheximide (10 µg/ml)	100 ± 10	90 ± 10
BCM (4 µl) + cycloheximide (10 µg/ml)	100 ± 10	95 ± 10

[a]100% = 170 ± 18 pmol/min/mg of protein (activity; substrate was 10 µmol/l [^{3}H]AEA) or 0.350 ± 0.040 absorbance units at 405 nm (content) respectively.
*$P < 0.01$ versus controls ($P > 0.05$ in all other cases).
MAFP = methyl-arachidonoyl fluorophosphonate.

Table II. Effect of trophoblast and inner cell mass (ICM) cells on fatty acid amide hydrolase (FAAH) activity

Conditioned medium added (4 µl/100 µl)	FAAH activity[a]
None	170 ± 18 (100)
Trophoblast-derived cells	785 ± 75 (462)*
ICM cells	301 ± 30 (177)*
Uterine fluid	152 ± 16 (89)
STO cells	153 ± 16 (90)

Values in parentheses are percentages.
The effect of conditioned media in which different cell types had been cultured for 2 h was assayed on mouse uterine FAAH. Also the effect of uterine fluid from pregnant mice (4 µl/100 µl) and of embryonic fibroblasts (STO cells) was assessed.
[a]Expressed as pmol/min/mg of protein (substrate was 10 µmol/l [^{3}H]AEA).
*$P < 0.01$ versus controls ($P > 0.05$ in all other cases).

without affecting the corresponding protein level, whereas the protein synthesis inhibitor cycloheximide (10 µg/ml) affected neither FAAH activity nor protein content (Table I).

In order to determine whether the FAAH activator was produced by trophoblast or ICM cells, we separated trophoblast from ICM of early blastocysts. The results showed that CM obtained from trophoblast and ICM cells possess the ability to enhance FAAH activity (Table II). Interestingly, the ability of CM from trophoblast cells to enhance FAAH activity was ~2-fold and ~2.5-fold higher than that of blastocysts or ICM cells respectively. Finally, we found that uterine fluid did not contain any FAAH activator, neither did CM obtained from STO cell monolayers (Table II).

BCM does not affect PLD activity, AMT activity or CBR binding in the uterus

The activity of phospholipase D (PLD) in mouse uterine extracts was not affected by BCM (Table III), under optimal conditions for the *N*-acyl-phosphatidylethanolamines (NAPE)-hydrolysing PLD (Moesgaard *et al*., 2000), as modified by Gubellini *et al*. (2002).

This is worth noting, because NAPE-hydrolysing PLD activity is considered a checkpoint in AEA synthesis, even though the lack of specific inhibitors makes it difficult to assess conclusively the contribution of this enzyme to AEA metabolism (Hansen *et al*., 2000; Moesgaard *et al*., 2000; Gubellini *et al*., 2002). To our knowledge, this is the first demonstration of an active NAPE-hydrolysing PLD in mouse uterus. The activity of uterine AEA membrane transporter (AMT) was not affected by treatment with BCM, but was significantly inhibited by 10 µmol/l AM404 (Table III), a selective AMT inhibitor (Piomelli *et al*., 2000). Moreover, the binding of [^{3}H]CP55,940 to CB receptors in mouse uterus was fully displaced by 10 µmol/l SR141716, but not by 10 µmol/l SR144528 (Table III). BCM had no affect on the binding (Table III). As SR141716 and SR144528 are CB1 and CB2 receptor antagonists respectively (Pertwee and Ross, 2002), the data are consistent with the presence of CB1, but not CB2, receptors in mouse uterus (Das *et al*., 1995). Finally, the activity of uterine PLD or AMT, and the binding to uterine CB receptors, were not affected by STO cell-conditioned media under the same experimental conditions used to test the effects of BCM (Table III).

Lipid nature of FAAH activator and effect of blastocyst-derived factors on uterine FAAH

In an attempt to clarify the molecular nature of the 'FAAH activator' released by blastocysts, we investigated the effect of several enzyme digestions of BCM and that of various factors known to be released by blastocysts (Psychoyos *et al*., 1995; Ammit and O'Neill, 1997; Ahmed *et al*., 1998) on uterine FAAH activity. Treatment of BCM with DNAse I, RNAse A, phospholipases (PL) A_2, C and D, α-chymotrypsin, bromelain, papain or pepsin A (10 IU each) did not affect its ability to enhance FAAH, either in tissue homogenates or in intact uterine horns, whereas trypsin (10 IU) further increased and lipase (10 IU) almost completely abolished such activity (Figure 1C). Treatment of uterine extracts or intact horns with trypsin or lipase did not affect FAAH activity under the same experimental conditions (not shown).

Platelet-activating factors (PAF)-16 and -18 were found to inhibit FAAH in tissue homogenates in a dose-dependent manner. Kinetic analysis of the inhibition indicated that PAF-16 and -18 acted as non-competitive inhibitors of FAAH, with K_i of ~3 µmol/l and ~2 µmol/l respectively (Figure 2A, and data not shown). Remarkably, FAAH activity of intact uterine horns was not affected by PAF-16 or -18 under the same experimental conditions (Figure 2B). Furthermore, none of the blastocyst-derived eicosanoids, i.e. leukotriene B_4 or prostaglandins E_2 and $F_2\alpha$, affected FAAH activity in uterine homogenates or intact tissues, when used at concentrations as high as 1 µmol/l (Figure 2B).

Discussion

Here, we have shown that mouse blastocysts rapidly (within 30 min of culture) release (a) compound(s), which increase(s) by ~2.5-fold the activity, but not the expression, of FAAH in mouse uterus. This 'FAAH activator' does not affect other proteins of the endocannabinoid system, and cannot be identified with any compound known to be released by blastocysts. It can be suggested that this activator may be instrumental in protecting blastocysts against the noxious effects of uterine endocannabinoids during implantation. The observation that only live blastocysts can release the activator of FAAH (Figure 1B), while embryonic fibroblasts (STO cells) cannot (Table II), seems to support the hypothesis of a specific cross-talk between blastocyst and receptive uterus, affecting the level of AEA. The release of the activator, besides being specific for blastocysts, seems to correlate with the competence of these cells to implant: late blastocysts > early blastocysts > morulae. In particular, the ability to produce the FAAH activator develops in the blastocyst independently of the influence of the uterine environment, as shown by the finding that blastocysts isolated from the uterus or obtained in culture from 2-cell embryos have a similar ability to secrete this compound in the medium. Interestingly, both ICM cells and trophoblasts (each used at 25/well), were able to release *in vitro* the FAAH activator, while the

Table III. Effect of conditioned media in which early blastocysts (BCM) or embryonic fibroblasts (STO cells, STO-CM) had been cultured for 2 h on phospholipase D (PLD), anandamide membrane transporter (AMT) and cannabinoid receptor (CBR) binding in mouse uterine horns

Compound added	PLD activity[a]	AMT activity[b]	CBR binding[c]
None	240 ± 25 (100)	115 ± 10 (100)	800 ± 75 (100)
BCM (4 µl/100 µl)	250 ± 25 (104)	120 ± 10 (104)	775 ± 80 (97)
STO-CM (4 µl/100 µl)	240 ± 25 (100)	104 ± 10 (90)	800 ± 75 (100)
AM404 (10 µmol/l)	ND	25 ± 4 (22)*	ND
SR141716 (10 µmol/l)	ND	ND	128 ± 14 (16)*
SR144528 (10 µmol/l)	ND	ND	736 ± 75 (92)

Values in parentheses are percentages.
[a]Expressed as pmol/min/mg of protein (substrate was 10 µmol/l 1,2-dioleoyl-3-phosphatidyl[2-^{14}C]ethanolamine).
[b]Expressed as pmol/min/mg of protein (substrate was 400 nmol/l [^{3}H]AEA).
[c]Expressed as fmol/mg of protein (ligand was 400 pmol/l [^{3}H]CP55,940).
ND = not determined.
*$P < 0.01$ versus controls ($P > 0.05$ in all other cases).

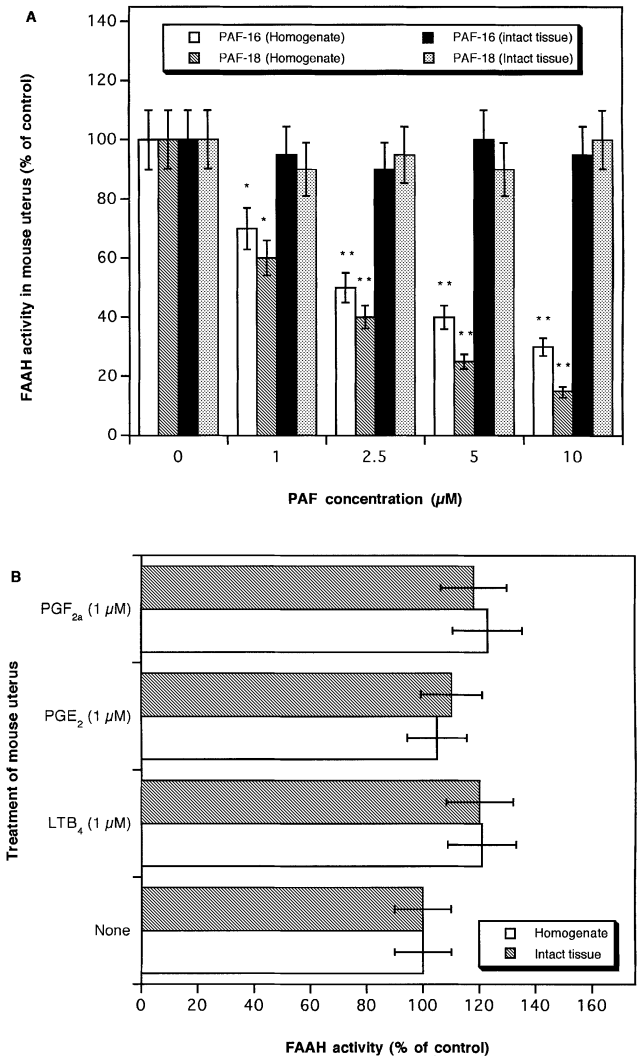

Figure 2. Effect of blastocyst-derived signals on uterine fatty acid amide hydrolase (FAAH). (**A**) Platelet-activating factors (PAF)-16 and -18 dose-dependently inhibited FAAH activity in mouse uterine extracts, but not in intact horns. (**B**) Leukotrienes B_4 (LTB_4) or prostaglandins (PG) E_2 and $F_2\alpha$ did not affect mouse uterine FAAH, either in tissue homogenates or in intact horns. In both panels, 100% = 170 ± 18 pmol/min/mg of protein. *$P < 0.05$, **$P < 0.01$ versus controls ($P > 0.05$ in all other cases).

contribution of uterus-derived factor(s) could be ruled out. Trophoblast cells appear to possess the greatest ability to produce the FAAH activator, in keeping with their critical role in embryo implantation. On the other hand, the lack of FAAH activator in uterine fluid of pregnant female may indicate that this compound is diluted in such a milieu or during flushing. At any rate, it demonstrates that the activator present in BCM derives from blastocysts, which are likely to be the major source of this substance. Moreover, these findings suggest that the action of such an activator may be important locally at the blastocyst implantation site rather than in the rest of the uterus.

Successful implantation is the result of an intimate cross-talk between the blastocyst and a receptive uterus (Paria *et al*., 1993). AEA might be critical in regulating the 'window' of implantation by synchronizing trophoblast differentiation and uterine preparation to the receptive state. This hypothesis is consistent with the observation that low levels of cannabinoid receptor agonists accelerate trophoblast differentiation and outgrowth, while higher doses inhibit trophoblast differentiation (Wang *et al*., 1999). Along these lines, higher levels of AEA found in non-receptive uterus correlate well with the developmental arrest, as well as with the observation that *in vitro* AEA inhibits embryo development and zona-hatching of blastocysts (Paria *et al*., 1996; Yang *et al*., 1996; Schmid *et al*., 1997) and induces apoptosis in these embryos (Maccarrone *et al*., 2000b). While mouse blastocysts have active AMT and FAAH, suggesting that AEA degradation might be instrumental in preventing its noxious effects on developing embryos (Maccarrone *et al*., 2000b), mouse uterus contains the highest levels of AEA detected in mammalian tissues to date (Schmid *et al*., 1997). Therefore, it might be advantageous to the embryo to reduce AEA levels locally by stimulating uterine FAAH, which is confined to the epithelium (Maccarrone *et al*., 2000b). To date, a FAAH activator has never been reported. Of course, a suitable 'activator' of uterine FAAH should be rapid, selective towards its target enzyme and be able to reach the inside intact cells, by crossing easily the plasma membrane barrier. The blastocyst-derived 'FAAH activator' described here appears to fulfil these requirements (Figure 1A–C and Table III). A lipid nature of this activator, suggested by the neutralizing effect of lipase (Figure 1C) and by the lack of effect on FAAH expression (Table I), seems to require that the activator interacts directly with the enzyme. As a matter of fact, a direct interaction is likely to occur between a lipid and a lipid-degrading enzyme. Moreover, it seems compatible with the potentiating effect of trypsin on the ability of BCM to enhance FAAH (Figure 1C). In fact, it is conceivable that a lipid compound released into an aqueous solution

needs a protein carrier, which might be cleaved at a specific site by trypsin, thus releasing higher amounts of activator, and/or possibly in a faster manner. In addition, the data on intact uteri suggest that the activator has the unprecedented ability to diffuse through the myometrium and endometrial stroma to reach the luminal epithelium where FAAH is localized, and to remain bound to the enzyme during processing of the tissue. Also these features seem to speak in favour of a lipid nature of the activator, suggesting that it may be retained into a lipid-binding pocket of FAAH.

In an attempt to identify the FAAH activator released by blastocysts, we checked the effect on FAAH of signals known to be released by blastocysts. They include PAF, leukotrienes and prostaglandins (Psychoyos *et al*., 1995; Ammit and O'Neill, 1997; Ahmed *et al*., 1998), which may act as triggers of signal transduction pathways (Dearn *et al*., 2000) and as controllers of blastocyst transport to the uterus (Velasquez *et al*., 2001) in humans. However, none of these compounds was able to activate mouse uterine FAAH (Figure 2). Two types of PAF, containing either palmitate (PAF-16) or stearate (PAF-18), were non-competitive inhibitors of FAAH in homogenates but not in intact uteri (Figure 2A), thus suggesting that the signalling pathways triggered by the binding of PAF to its receptor (Ammit and O'Neill, 1997; Ahmed *et al*., 1998; Dearn *et al*., 2000) did not affect FAAH. The possible significance of this effect on FAAH, and hence on AEA homeostasis, in pathophysiological conditions remains to be elucidated. It should be pointed out that blastocyst-conditioned medium is only available in tiny amounts, far below the quantities needed for the biochemical purification of the FAAH activator. Despite the incomplete characterization, the release by blastocysts of (a) compound(s) able to enhance FAAH activity in intact uterine cells appears to be a very interesting outcome of this investigation, which further strengthens the concept that the preimplantation mammalian embryo produces factors which can influence its own development (Lane and Gardner, 1992). In this context, the critical role of FAAH in the cross-talk between blastocyst and receptive uterus described in this study seems to hold true for mice with a normal genetic background, but apparently not for those lacking the *FAAH* gene. In fact, the latter animals are fertile, and are indistinguishable from wild-type littermates also in many other aspects, such as body weight, locomotion and overt behaviour, normally under control of the endocannabinoid system (Cravatt *et al*., 2001). Overall, it can be suggested that compensatory mechanisms take place in mice lacking FAAH, in analogy with previous observations in CB1 receptor knockout animals (Ledent *et al*., 1999).

Acknowledgements

We wish to thank Drs Monica Bari, Valeria Gasperi and Mara Lobascio for their skilful assistance in cell culture and treatment. This study was supported by Ministero dell'Istruzione, dell'Università e della Ricerca (Cofin'2002 to M.M.), Italy.

References

Ahmed A, Dearn S, Shams M, Li XF, Sangha RK, Rola-Pleszczynski M, Jiang J (1998) Localization, quantification, and activation of platelet-activating factor receptor in human endometrium during the menstrual cycle: PAF stimulates NO, VEGF, and FAKpp125. FASEB J 12,831–843.

Ammit AJ and O'Neill C (1997) The role of albumin in the release of platelet-activating factor by mouse preimplantation embryos in vitro. J Reprod Fertil 109,309–318.

Battista N, Bari M, Finazzi-Agrò A and Maccarrone M (2002) Anandamide uptake by synaptosomes from human, mouse and rat brain: inhibition by glutamine and glutamate. Lipids Health Dis 1,1.

Cravatt BF, Demarest K, Patricelli MP, Bracey MH, Giang DK, Martin BR and Lichtman AH (2001) Supersensitivity to anandamide and enhanced endogenous cannabinoid signaling in mice lacking fatty acid amide hydrolase. Proc Natl Acad Sci USA 98,9371–9376.

Das SK, Paria BC, Chakraborty I and Day SK (1995) Cannabinoid ligand-receptor signaling in the mouse uterus. Proc Natl Acad Sci USA 92,4332–4336.

Dearn S, Rahman M, Lewis A, Ahmed Z, Eggo MC and Ahmed A (2000) Activation of platelet-activating factor (PAF) receptor stimulates nitric oxide (NO) release via protein kinase C-alpha in HEC-1B human endometrial epithelial cell line. Mol Med 6,37–49.

De Petrocellis L, Melck D, Bisogno T and Di Marzo V (2000) Endocannabinoids and fatty acid amides in cancer, inflammation and related disorders. Chem Phys Lipids 108,191–209.

Deutsch DG, Goligorsky MS, Schmid PC, Krebsbach RJ, Schmid HHO, Das SK, Dey SK, Arreaza G, Thorup C, Stefano G and Moore LC (1997) Production and physiological actions of anandamide in the vasculature of the rat kidney. J Clin Invest 100,1538–1546.

Fowler CJ, Jonsson K-O and Tiger G (2001) Fatty acid amide hydrolase: biochemistry, pharmacology and therapeutic possibilities for an enzyme hydrolyzing anandamide, 2-arachidonoylglycerol, palmitoyethanolamide and oleamide. Biochem Pharmacol 62,517–526.

Giang DK and Cravatt BF (1997) Molecular characterization of human and mouse fatty acid amide hydrolase. Proc Natl Acad Sci USA 94,2238–2242.

Gubellini P, Picconi B, Bari M, Battista N, Calabresi P, Centonze D, Bernardi G, Finazzi-Agrò A and Maccarrone M (2002) Experimental parkinsonism alters endocannabinoid degradation: implications for striatal glutamatergic transmission. J Neurosci 22,6900–6907.

Hall W and Solowij N (1998) Adverse effects of cannabis. Lancet 352,1611–1616.

Hansen HS, Moesgaard B, Hansen HH and Petersen G (2000) N-Acylethanolamines and precursor phospholipids: relation to cell injury. Chem Phys Lipids 108,135–150.

Hanus L, Abu-Lafi S, Fride E, Breuer A, Vogel Z, Shalev DE, Kustanovich I and Mechoulam R (2001) 2-Arachidonyl glyceryl ether: an endogenous agonist of the cannabinoid CB1 receptor. Proc Natl Acad Sci USA 98,3662–3665.

Hillard CJ and Jarrahian A (2000) The movement of N-arachidonoylethanolamine (anandamide) across cellular membranes. Chem Phys Lipids 108,123–134.

Hillard CJ, Edgemond WS, Jarrahian A and Campbell WB (1997) Accumulation of N-arachidonoylethanolamide (anandamide) into cerebellar granule cells occurs via facilitated diffusion. J Neurochem 69,631–638.

Howlett AC and Mukhopadhyay S (2000) Cellular signal transduction by anandamide and 2-arachidonoylglycerol. Chem Phys Lipids 108,53–70.

Kunos G, Járai Z, Batkai S, Goparaju SK, Ishac EJ, Liu J, Wang L and Wagner JA (2000) Endocannabinoids as cardiovascular modulators. Chem Phys Lipids 108,159–168.

Lane M and Gardner DK (1992) Effect of incubation volume and embryo density on the development and viability of mouse embryos in vitro. Hum Reprod 7,558–562.

Ledent C, Valverde O, Cossu G, Petitet F, Aubert J-F, Beslot F, Böhme GA, Imperato A, Pedrazzini T, Roques BP, Vassart G, Fratta W and Parmentier M (1999) Unresponsiveness to cannabinoids and reduced addictive effects of opiates in CB_1 receptor knockout mice. Science 283,401–404.

Maccarrone M, Bari M and Finazzi-Agrò A (1999) A sensitive and specific radiochromatographic assay of fatty acid amide hydrolase. Anal Biochem 267,314–318.

Maccarrone M, Valensise H, Bari M, Lazzarin N, Romanini C and Finazzi-Agrò A (2000a) Relation between decreased anandamide hydrolase concentrations in human lymphocytes and miscarriage. Lancet 355,1326–1329.

Maccarrone M, De Felici M, Bari M, Klinger F, Siracusa G and Finazzi-Agrò A (2000b) Down-regulation of anandamide hydrolase in mouse uterus by sex hormones. Eur J Biochem 267,2991–2997.

Maccarrone M, Lorenzon T, Bari M, Melino G and Finazzi-Agrò A (2000c) Anandamide induces apoptosis in human cells via vanilloid receptors Evidence for a protective role of cannabinoid receptors. J Biol Chem 275,31938–31945.

Maccarrone M, Valensise H, Bari M, Lazzarin N, Romanini C and Finazzi-Agrò A (2001) Progesterone up-regulates anandamide hydrolase in human lymphocytes Role of cytokines and implications for fertility. J Immunol 166,7183–7189.

Maccarrone M, Bisogno T, Valensise H, Lazzarin N, Fezza F, Manna C, Di Marzo V and Finazzi-Agrò A (2002) Low fatty acid amide hydrolase and

high anandamide levels are associated with failure to achieve an ongoing pregnancy after IVF and embryo transfer. Mol Hum Reprod 8,188–195.

Mani SK, Mitchellm A and O'Malley BW (2001) Progesterone receptor and dopamine receptors are required in Δ^9-tetrahydrocannabinol modulation of sexual receptivity in female rats. Proc Natl Acad Sci USA 98,1249–1254.

Mechoulam R and Hanus L (2000) A historical overview of chemical research on cannabinoids. Chem Phys Lipids 108,1–13.

Melck D, De Petrocellis L, Orlando P, Bisogno T, Laezza C, Bifulco M and Di Marzo V (2000) Suppression of nerve growth factor Trk receptors and prolactin receptors by endocannabinoids of human breast and prostate cancer. Endocrinology 141,118–126.

Moesgaard B, Petersen G, Jaroszewski JW and Hansen HS (2000) Age dependent accumulation of *N*-acyl-ethanolamine phospholipids in ischemic rat brain A ^{31}P NMR and enzyme activity study. J Lipid Res 41,985–990.

Paria BC and Dey SK (2000) Ligand-receptor signaling with endocannabinoids in preimplantation embryo development and implantation. Chem Phys Lipids 108,211–220.

Paria BC, Huet-Hudson Y and Dey SK (1993) Blastocyst's state of activity determines the 'window' of implantation in the receptive mouse uterus. Proc Natl Acad Sci USA 90,10159–10162.

Paria BC, Deutsch DD and Dey SK (1996) The uterus is a potential site for anandamide synthesis and hydrolysis: differential profiles of anandamide synthase and hydrolase activities in the mouse uterus during the periimplantation period. Mol Reprod Dev 45,183–192.

Paria BC, Zhao X, Wang J, Das SK and Dey SK (1999) Fatty-acid amide hydrolase is expressed in the mouse uterus and embryo during the periimplantation period. Biol Reprod 60,1151–1157.

Paria BC, Song H, Wang X, Schmid PC, Krebsbach RJ, Schmid HH, Bonner TI, Zimmer A and Dey SK (2001) Dysregulated cannabinoid signaling disrupts uterine receptivity for embryo implantation. J Biol Chem 276,20523–20528.

Parolaro D, Massi P, Rubino T and Monti E (2002) Endocannabinoids in the immune system and cancer. Prostagl Leukot Essent Fatty Acids 66,319–332.

Pertwee RG and Ross RA (2002) Cannabinoid receptors and their ligands. Prostagl Leukot Essent Fatty Acids 66,101–121.

Piomelli D, Giuffrida A, Calignano A and Rodriguez de Fonseca F (2000) The endocannabinoid system as a target for therapeutic drugs. Trends Pharmacol Sci 21,218–224.

Psychoyos A, Nikas G and Gravanis A (1995) The role of prostaglandins in blastocyst implantation. Hum Reprod 10,30–42.

Schmid PC, Paria BC, Krebsbach RJ, Schmid HHO and Dey SK (1997) Changes in anandamide levels in mouse uterus are associated with uterine receptivity for embryo implantation. Proc Natl Acad Sci USA 94,4188–4192.

Schuel H, Burkman LJ, Lippes J, Crickard K, Forester E, Piomelli D and Giuffrida A (2002) N-Acylethanolamines in human reproductive fluids. Chem Phys Lipids 121,211–227.

Sugiura T and Waku K (2000) 2-Arachidonoylglycerol and the cannabinoid receptors. Chem Phys Lipids 108,89–106.

Ueda N, Puffenbarger RA, Yamamoto S and Deutsch DG (2000) The fatty acid amide hydrolase (FAAH). Chem Phys Lipids 108,107–121.

Velasquez LA, Maisey K, Fernandez R, Valdes D, Cardenas H, Imarai M, Delgado J, Aguilera J and Croxatto HB (2001) PAF receptor and PAF acetylhydrolase expression in the endosalpinx of the human Fallopian tube: possible role of embryo-derived PAF in the control of embryo transport to the uterus. Hum Reprod 16,1583–1587.

Wang J, Paria BC, Dey SK and Armant DR (1999) Stage-specific excitation of cannabinoid receptor exhibits differential effects on mouse embryonic development. Biol Reprod 60,839–844.

Yang Z-M, Paria BC and Dey SK (1996) Activation of brain-type cannabinoid receptors interferes with preimplantation mouse embryo development. Biol Reprod 55,756–761.

Submitted on November 19, 2003; accepted on December 2, 2003