ISSN 1669-5402 (Print) ISSN 1669-5410 (Online)

Physiological Mini-Reviews

Edited by the Argentine Physiological Society.

Vol. 3, N° 1, August-September 2007.

http://www.mini.reviews.safisiol.org.ar

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[ISSN 1669-5402 (Print); ISSN 1669-5410 (Online)]

Edited by the **Argentine Physiological Society**

Journal address: Sociedad Argentina de Fisiología, Universidad Favaloro, Solís 453 (1078),
Ciudad de Buenos Aires Argentina.
Tel.-Fax: (54) (0)11 43781151
http://www.mini.reviews.safisiol.org.ar

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(Resol. IGJ 763-04)

THE ROLE OF ANANDAMIDE DURING PREGNANCY. A SHORT TALE ABOUT THE ENDOCANNABINOID SYSTEM.

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Abbreviated title: Intracellular Ionic and ATP regulation of the Na^+/Ca^{2+} exchanger.

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INTRODUCTION.

The success of any species depends on its reproductive efficiency. Sexual procreation is initiated by interactions between a sperm and an egg leading to fertilization. The fertilized egg (embryo) undergoes several mitotic cell divisions, ultimately producing the blastocyst. The nurturing of an offspring within the body and production of a live birth is an enduring task, requiring safeguard regulatory systems at various critical steps. At the moment, there is still a significant knowledge gap in understanding the mechanisms by which a successful pregnancy is achieved. It is difficult to define the hierarchical landscape of the molecular pathways during human pregnancy, because of experimental difficulties and ethical restrictions on research with human embryos. It is hoped that experiments on mice and other animal models that bear certain reproductive similarities with humans combined with those feasible experiments in humans would generate meaningful information to address this critical issue. A deeper insight into these processes will help to generate new ideas and concepts for improving fertility and pregnancy-associated health issues in humans. During the last years, several studies have provided evidence that lipid mediators are important signaling molecules in coordinating a series of events during pregnancy. Increasing evidence points toward the pathophysiological significance of endocannabinoids, a group of bioactive lipid-signaling molecules, in both female and male fertility.

THE ENDOCANNABINOID SYSTEM.

Endocannabinoids are unsaturated fatty acid derivatives which act as endogenous ligands for cannabinoid receptors and mimic the effects of natural existing cannabinoids, as Δ^9-tetrahydrocannabinol (THC), the main psychoactive component of marijuana. The first product of this group, *anandamide* (AEA, N-arachidonylethanolamide), was isolated from the porcine brain in 1992 (Devane *et al.* 1992). The name was coined from the Sanskrit word ‘‘ananda’’ meaning bliss. Since then a number of fatty acid derivatives that belong to this group has been isolated (**Figure 1**). These include sn-2 arachidonyl-

glycerol, 2-AG-ether (noladin ether) and O-arachidonoylethanolamine (virodhamine), an "inverted AEA".

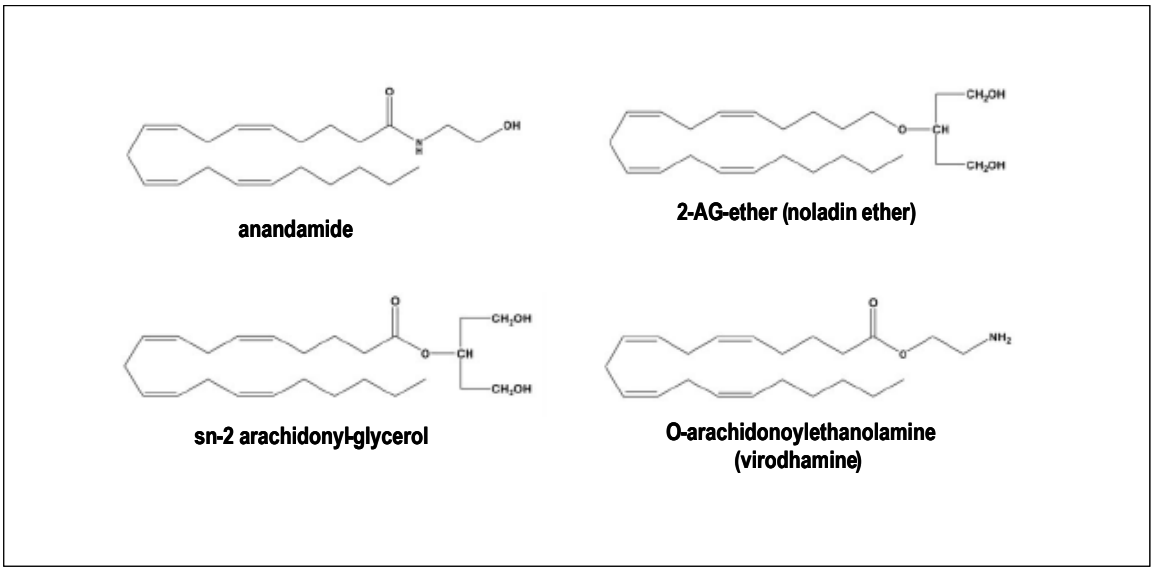

Figure 1. Chemical structures of endogenous cannabinoids.

The discovery of AEA caused intense investigation regarding its biosynthetic pathway. Enzymatic synthesis of AEA was first described in bovine brain as the conjugation of arachidonic acid with ethanolamine (Devane and Axelrod 1994). Recently, it has been reported that AEA could be also synthezised by two different phospholipases: N-arachidonylphosphatidylethanolamine phospholipase-D (NAPE-PLD) (Ueda *et al*. 2005) and a subtype of phopholipase (PLC) (Liu *et al*. 2006). The biological activity of AEA at CB receptors is terminated by its removal from the extracellular space, which occurs through a two-step process: cellular uptake by a high affinity transporter, followed by intracellular degradation by a fatty acid amide hydrolase (FAAH) (Cravatt *et al*. 1996). Several properties of a selective AEA membrane transporter (AMT) have been characterized, although its molecular structure remains unknown. In fact, there is controversy regarding its existence and the mechanism by which AEA is taken up by cells is currently being debated.

It has been found that AEA mainly exerts its effects through the activation of specific cannabinoid receptors (CB-R) located in the surface of target cells known as CB1 and CB2, typical of the G protein-coupled receptor superfamily (Howlett *et al*. 2002). CB1 is expressed in the brain and many other tissues: peripheral nerves, spleen, vascular endothelial muscle cells, eye and in reproductive organs such as, placenta, uterus, oviduct, testis and vas deferent. CB2 was first described in spleen and immune system cells. Besides, AEA could bind to the cytosolic domain of another type of receptors known as TRPV (transient receptor potential vanilloid), leading to the concept that AEA, besides being an endocannabinoid, is also a true "endovanilloid" (Van Der Stelt *et al*. 2004). A schematic view of AEA metabolic pathway and way of action is shown in **Figure 1B** (**Figure 2**).

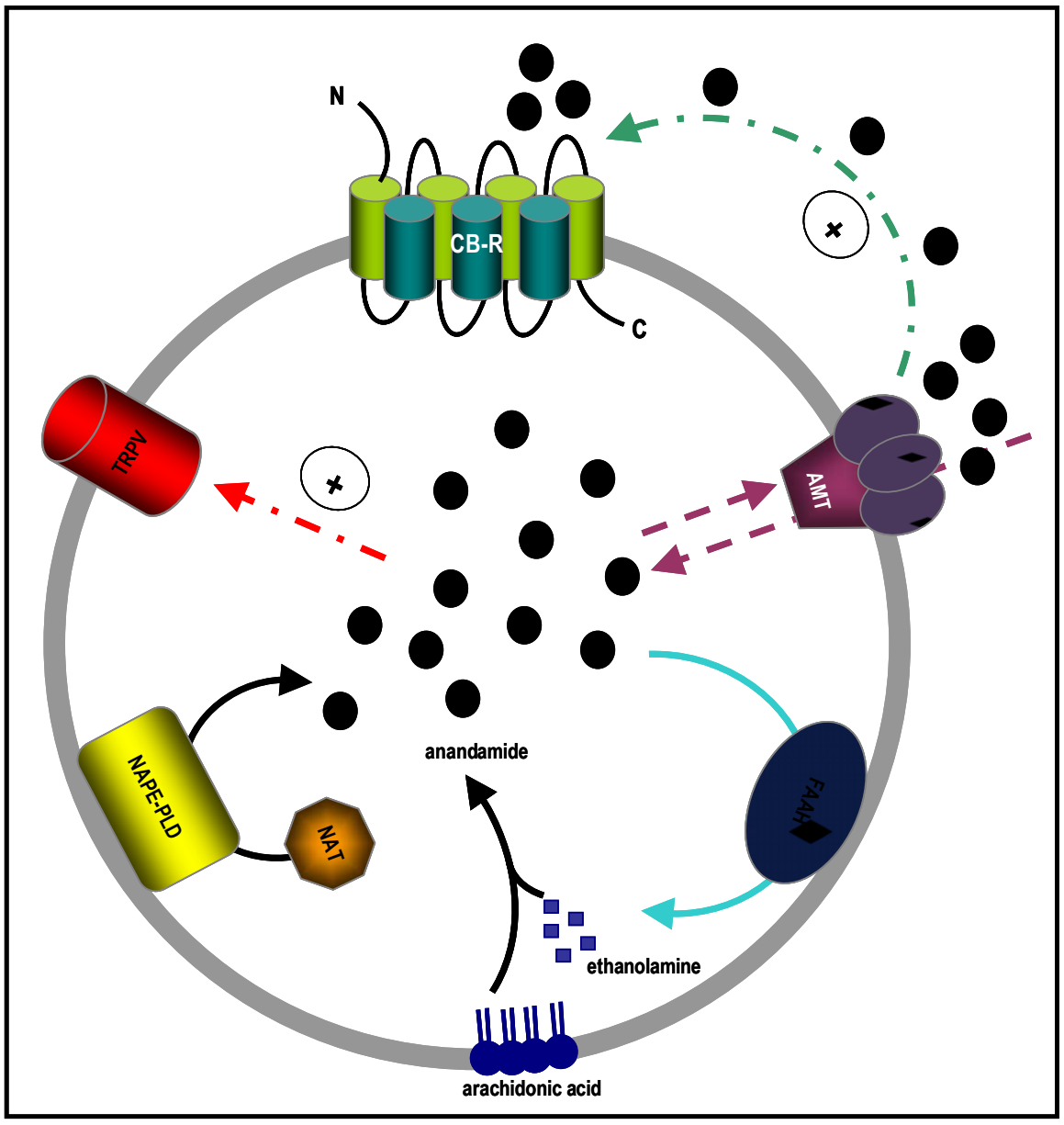

Figure 2. Anandamide metabolic pathway. *The synthesis of AEA from membrane N-arachidonoylphosphatidylethanolamines is catalyzed by the sequential activity of NAT and NAPE-PLD or by the condensation of arachidonic acid and ethanolamine. AEA is transported in both directions through the cell membrane by a putative transporter (AMT) and, once taken up, is hydrolyzed by FAAH to ethanolamine and arachidonic acid. The main targets of AEA are CB1 and CB2 receptors (CB-R), showing an extracellular binding site, and vanilloid receptors (TRPV), showing an intracellular binding site.*

ANANDAMIDE AND MALE FERTILITY.

Although there is quite evidence that chronic administration of THC is correlated with male infertility, the role of the endocannabinoid system is still largely unexplored (for references see Wang *et al.* 2006a).

CB1 expression has been described in Leydig cells (Gye *et al*. 2005) associated with testosterone secretion. Because Sertoli cells are involved in the regulation of germ cell development, their ability to bind and degrade AEA seems important in controlling the

spermatogenic output. As AEA can serve as a proapoptotic factor, it may also be involved in the survival and death of Sertoli cells (Orth *et al*. 1998).

Follicular, oviductal and seminal fluids contain significant concentrations of endocannabinoids (Schuel *et al*. 2002a), suggesting that these lipids may influence important processes involved in the control of sperm/egg functions and fertilization. Recently, it has been reported that human sperm express CB1 and that its activation inhibits capacitation and acrosome reaction (Rossato *et al*. 2005). In addition, R(+)-methanandamide, a potent and metabolically stable AEA analogue, modulates hyperactivated human spermatozoa motility (Schuel *et al*. 2002b). Boar spermatozoa have the biochemical machinery to bind (CB1 and TRPV1), synthesize (NAPE-PLD), and degrade (AMT and FAAH) AEA (Maccarrone *et al*. 2005) (**Figure 3**). It was also shown that activation of CB1 by R(+)-methanandamide inhibits capacitation while once the capacitation is completed, AEA stabilizes the acrosome membranes by activating TRPV1, thus reducing spontaneous acrosome reaction.

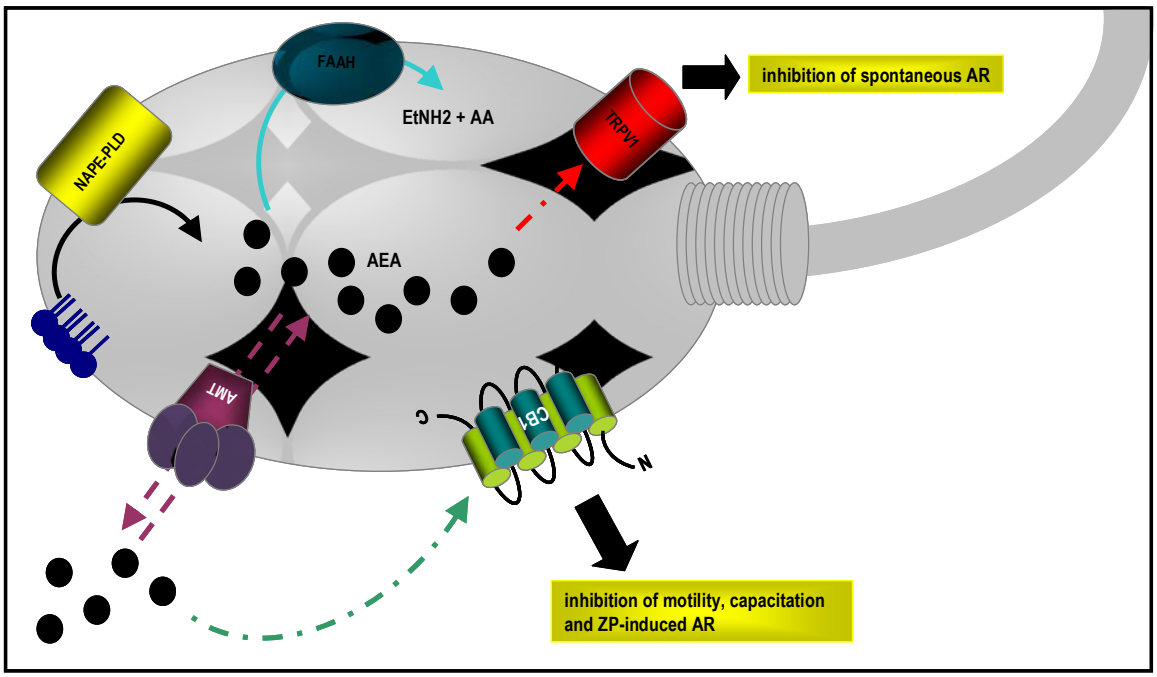

Figure 3. Anandamide in sperm function. *Binding of AEA to the extracellular site of CB1 leads to inhibition of sperm motility, capacitation, and zona pellucida (ZP)-induced acrosomal reaction (AR), without affecting spontaneous AR. In contrast, binding of AEA to the intracellular site of type-1 vanilloid receptors (TRPV1) inhibits spontaneous AR. Sperm also possess the AMT, the AEA-synthesizing phospholipase D (NAPE-PLD), and the AEA-hydrolyzing FAAH. FAAH cleaves AEA into ethanolamine (EtNH2) and arachidonic acid (AA).*

Spermatozoa attachment and release from oviductal reservoirs play a role not only in the temporal coordination of fertilization but also in assuring that the adequate number of spermatozoa arrives to the site of fertilization in a controlled way. In the mammalian oviduct, sperm hyperactivation takes place at the time of ovulation. Hyperactivated sperm initiates a vigorous swimming pattern characterized by a high flagellar beating and coincides with the ability of sperm to be released from oviductal reservoir. Since several evidences indicate that mammalian sperm possess CB-R and that AEA signaling might regulate sperm functions required for fertilization (Schuel *et al*. 2002b), we hypothesized

that in the oviduct, AEA could be involved in the regulation of timely sperm escape from oviductal reservoirs allowing selected sperm to migrate to the fertilization site. We have observed for the first time that AEA is capable to induce sperm release from oviductal epithelial cells in bovines (Rapanelli *et al*. 2006). These evidences are supported by the fact that the bull spermatozoa as well as the bovine oviduct express CB1 and CB2 and that CB1 mediates AEA effect. Further investigations should be performed to elucidate whether AEA acts on the sperm and / or on oviductal cells to prevent sperm-oviduct interactions or to induce the release of the sperm bound to bovine oviductal epithelial cells. It is also necessary to understand which mechanisms regulate AEA secretion and which molecules are involved in AEA pathway.

ANANDAMIDE AND FEMALE FERTILITY.

It has been clearly demonstrated that endocannabinoid signaling profoundly influences female reproduction during early pregnancy. In mice, only CB1 is expressed in the oviduct and uterus, whereas both CB1 and CB2 are expressed in preimplantation embryos. However, we have observed that mice uteri during late gestation and rat uteri during the peri-implantation period express both CB1 and CB2 (Ribeiro *et al*. 2006, Franchi *et al*. 2005) Uterine AEA levels and blastocyst CB1 are coordinately downregulated with the attainment of uterine receptivity and blastocyst activation prior to implantation as opposed to their higher levels in delayed implanting uterus and dormant blastocysts (Paria *et al*. 2001, Schmid *et al*. 1997). These results suggest that lower levels, but not higher levels, of AEA and CB1 are beneficial to implantation. Indeed, AEA at low levels induces blastocyst activation by activating MAPK signaling while at higher levels inhibits blastocyst activation for implantation by blocking calcium signaling via CB1 (Wang *et al*. 2003). The role of CB2 in the early embryo is yet to be defined. Also, it has been demonstrated that uterine NAPE-PLD expression and activity change in a spatiotemporal manner determining AEA levels at the implantation site and during various states of uterine receptivity for implantation (Guo *et al*. 2005). We have observed that uterine anandamide synthesizing capacity coincides with this pattern and that it is regulated by progesterone and estradiol through blastocyst activation (Sordelli *et al*. 2006).

Last year, and collegues (2006b) showed that NAPE-PLD and FAAH regulate AEA levels during early pregnancy, and their coordinated expression in the mouse oviduct and preimplantation embryos suggest that there is a physiological AEA tone for normal development of preimplantation embryos and their timely homing into uterus for implantation. Collectively, these findings led to speculate that uterine anandamide and embryonic CB1 is one of the signaling pathways that determine the fate of embryo implantation and that under normal physiological settings, this basal AEA tone imparts an appropriate CB1 function that is crucial to early pregnancy events (**Figure 4**).

The results observed in mice, however, could not explain the underlying causes of pregnancy loss in a substantial number of CB1 knock out mice. Besides of implantation, timely transport of embryos from the oviduct into the uterus is another critical event during early gestation. Thus, normal oviductal embryo transport is prerequisite for on-time implantation. In 2004, Wang and collaborators showed that oviductal embryo transport during early pregnancy is coordinated by endocannabinoid signaling through oviductal CB1 in collaboration with adrenergic receptor signaling and that subfertility in *Cb1*–/– mice is due to oviductal retention of embryos.

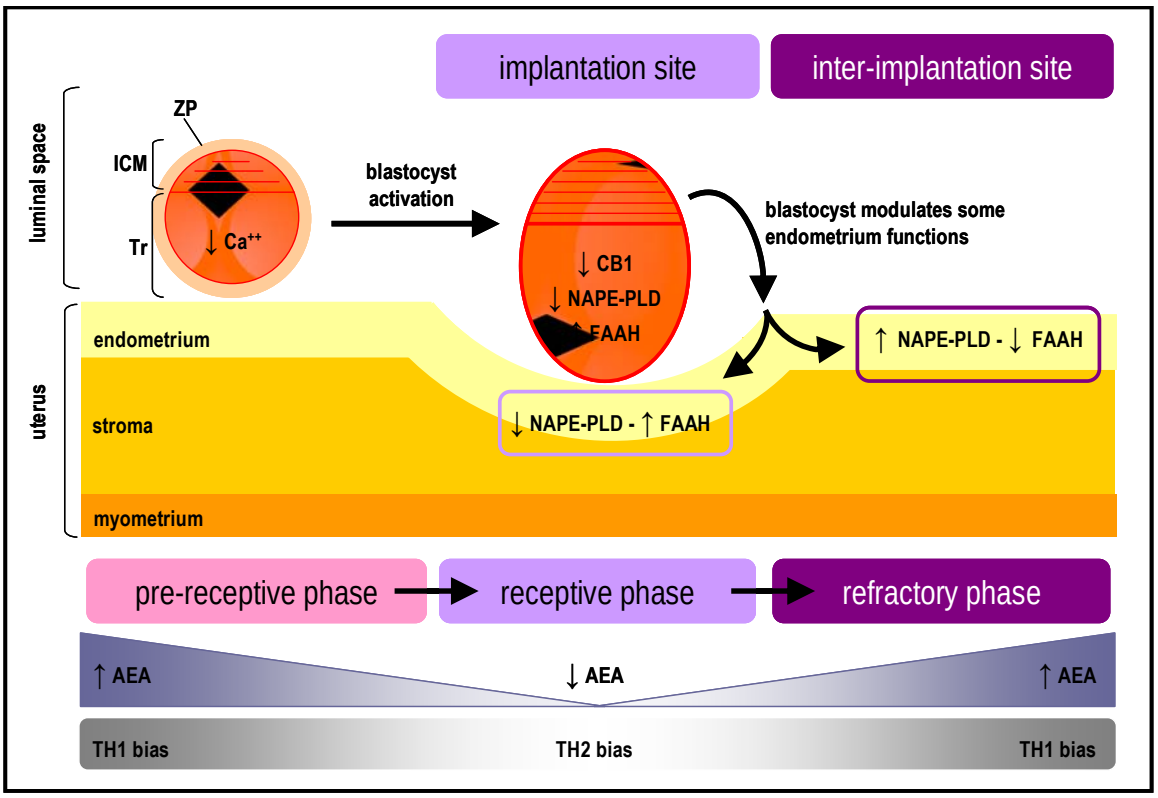

Figure 4. Anandamide signaling in blastocyst activation and implantation. *Evidence suggests that regulated levels of AEA in the receptive uterus and CB1 in activated blastocysts, are beneficial for implantation, whereas higher levels are detrimental to this process. This biphasic role of AEA is further supported by findings that AEA within a very narrow range regulates blastocyst activation and implantation. Uterine AEA levels conducive to implantation are primarily regulated by the coordinated expression and activity of NAPE-PLD and FAAH during early pregnancy. In addition, the implanting blastocyst down-regulates uterine NAPE-PLD expression, but enhances uterine FAAH activity, thus contributing to rapid turnover of AEA at the implantation site. ICM: inner cell mass, Tr: trophectoderm, ZP: zona pellucida.*

There is evidence for the role of peripheral lymphocytes in embryo implantation and successful pregnancy in humans. In fact, normal gestation is based on an early immunological adaptation that involves peripheral T lymphocytes in pregnant women. In this respect, lymphocyte FAAH has been shown to influence pregnancy outcome by regulating AEA level at the fetalmaternal interface, which appears to interfere with the lymphocyte-dependent cytokine network. FAAH expression in T lymphocytes has been shown to be regulated by Th1/Th2 cytokines: anti-inflammatory cytokines enhance FAAH activity, whereas pro-inflammatory cytokines attenuate its activity. An association of spontaneous pregnancy loss with elevated peripheral AEA levels in women (Maccarone *et al*. 2000) is consistent with the observations in mice. It is interesting to note that the peripheral AEA levels remain relatively low during implantation, whereas the levels increase before and during parturition in humans (Habayeb *et al*. 2004). The highest FAAH activity and the lowest AEA concentrations are observed on day 21 of the cycle, a period that temporally coincides with the putative window of uterine receptivity for implantation; binding to CB1 and activities of AMT and NAPE-PLD were similar in T cells at all stages of the ovulatory cycle. Also, CB1 activation inhibits human decidualization and promotes apoptosis of decidual cells *in vitro*, thus adding a new role of AEA in human pregnancy.

Previous results from our own indicate that lipopolisaccharide, an integral part of the outer membrane of Gram negative bacteria, is capable of producing embryonic resorption in mice due to NO increased production not only in uterus but also in decidua (Ogando *et*

al. 2003). Recent research has revealed that LPS induces AEA synthesis in murine macrophages. We have observed that AEA is also able of inducing embryonic resorption and embryonic growth retardation as well as an increment in NO production, suggesting that AEA migth be mediating lipopolisaccharide deleterious effect on implantation (Vercelli *et al*. 2006).

ANANDAMIDE AND TERM GESTATION.

Human pregnant myometrium expresses CB1 and CB2 (Dennedy *et al*. 2004). Both endogenous and exogenous cannabinoids exert a potent and direct relaxant effect on this tissue, which is mediated through the CB1 receptor. However the synthesis of AEA is increased in term mice uteri, the administration of CB1 antagonists during late gestation do not modify the duration of gestation, raising the question about the role of endocannabinoids during late gestation in the uterus (Franchi *et al.* 2005).

The placenta is the primary link between the mother and the conceptus and is essential for the growth and survival of the fetus. Abnormalities in placental formation and function could have a major influence on pregnancy outcome. The endocannabinoid system (AEA, CB1 and CB2, FAAH) is present in the rat placenta (Farina *et al*. 2006). AEA inhibits nitric oxide synthesis during late gestation through CB1 and CB2 receptors, suggesting that this endocannabinoid could be one of the endogenous molecules that regulate placental nitric oxide production during term pregnancy.

FUTURE DIRECTIONS.

Taken together, sperm function seems to be regulated by endocannabinoids that exert a dual stage-dependent effect. On one hand, AEA, present in both seminal plasma and uterine fluids, may prevent premature capacitation in freshly ejaculated sperm via a CB1-mediated mechanism for traveling along the uterine tract without any fertilizing potential. Conversely, a few hours later when sperm have reached the oviduct (a condition that corresponds to *in vitro* capacitation), this inhibitory brake becomes less stringent. It is speculated that spermatozoa are exposed to a progressively reduced concentration of AEA in the proximal female genital tract, and sperm capacitation occurs as a consequence of release from CB1 inhibition. The observation that the endocannabinoid system is operative in sperm adds a new dimension to the intricate endocannabinoid network regulating mammalian fertility. Overall, these findings present new perspectives to the understanding and treatment of male fertility problems.

Both the medicinal and the recreational use of marijuana are increasing worldwide. It is one of the most popular illicit drugs used by pregnant women, raising concern about marijuana's adverse effects during pregnancy. Maternal use of marijuana is associated with reduced birth weight as well as cognitive and memory deficits in offspring. These observations led to the studies concerning the role of endogenous cannabinoids in female reproduction. The reports about AEA signaling in the oviduct, have important implications for ectopic pregnancy in women because one major cause of tubal pregnancy is embryo retention in the fallopian tube. Embryo retention has become an issue of concern in light of the recent evidence that marijuana use or dependence in adults aged 18 and over in the United States has increased by 22% since 1991. With respect to early pregnancy, AEA

tight regulation regarding its synthesis and hydrolysis in the pregnant uterus further indicates that endocannabinoid ligand-receptor signaling plays an important role in implantation. The up-regulation of lymphocyte FAAH by profertility signals strengthens the speculation that this enzyme affects human fertility by modulating the AEA levels. The fact that CB receptors are present in women pregnant endometrium and that AEA modulates its contractility, highlights a possible role for endogenous cannabinoids during human parturition and pregnancy. These results also support the view that the use of exogenous cannabinoids during pregnancy is not linked independently with preterm labor.

However, the results obtained during the last years and reviewed here have to be analyzed carefully, taking into account that marijuana is composed by several substances different from THC and that a high proportion of pregnant women that consume marijuana also consume alcohol and other illicit drugs. Thus, the results shown for endocannabinoids should not be directly interpreted as the explanation for the way of action that marijuana exerts on pregnancy. Besides, the pathophysiological impact of other endocannabinoids or "endocannabinoid-like" compounds on various reproductive events warrants further investigation.

ACKNOWLEDGMENT

The authors thank Ramona Morales, Ana Inés Casella and Patricia Fernández for their technical support. Studies by the authors incorporated in this review were supported by FONCYT (PICT 2004 Nº10901) and CONICET (PIP Nº6127) to Dra. Ana María Franchi, FONCYT (PICT 2003 Nº0113398) to Dra. Silvina Pérez Martínez and FONCYT (PICT 2005 Nº35016) to Dra. Mariana Farina. We apologize for unintended omission of any relevant references.

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