Temporal and Spatial Distribution of the Cannabinoid Receptors (CB1, CB2) and Fatty Acid Amide Hydroxylase in the Rat Ovary

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Temporal and Spatial Distribution of the Cannabinoid Receptors (CB_1, CB_2) and Fatty Acid Amide Hydroxylase in the Rat Ovary

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ABSTRACT

Although the effects of Δ^9-tetrahydrocannabinol (THC) on ovarian physiology have been known for many decades, its mechanism of action in the rat ovary remains poorly understood. The effects of THC and endocannabinoids on many cell types appear to be mediated through the G-protein-coupled CB_1 and CB_2 receptors. Evidence also suggests that the concentration of the endocannabinoid anandamide is regulated by cellular fatty acid amide hydrolase (FAAH). Therefore, we examined the rat ovary for the presence of CB_1 and CB_2 receptors and FAAH. The CB_1 receptor was present in the ovarian surface epithelium (OSE), the granulosa cells of antral follicles, and the luteal cells of functional corpus luteum (CL). The granulosa cells of small preantral follicles, however, did not express the CB_1 receptor. Western analysis also demonstrated the presence of a CB_1 receptor. In both preantral and antral follicles, the CB_2 receptor was detected only in the oocytes. In the functional CL, the CB_2 receptor was detected in the luteal cells. FAAH was codistributed with CB_2 receptor in both oocytes and luteal cells. FAAH was also present in the OSE, subepithelial cords of the tunica albuginea (TA) below the OSE, and in cells adjacent to developing preantral follicles. Western analysis also demonstrated the presence of FAAH in oocytes of both preantral and antral follicles. Our observations provide potential explanation for the effects of THC on steroidogenesis in the rat ovary observed by earlier investigators and a role for FAAH in the regulation of ovarian anandamide. Anat Rec, 293:1425–1432, 2010. © 2010 Wiley-Liss, Inc.

Key words: cannabinoid receptors; FAAH; follicles; corpus luteum

Although the mechanism of signal transduction by cannabinoids is only now being teased out, the effects of Δ^9-tetrahydrocannabinol (THC) on female reproductive system have been known for decades. THC affects prenatal development (Dalterio and Bartke, 1981; Rosenkrantz et al., 1986; Abel et al., 1987), secretion of gonadotropins (Asch et al., 1979; Smith et al., 1979; Dalterio et al., 1983; Mendelson et al., 1986) and progesterone (Almirez et al., 1983), and menstrual cycle (Asch et al., 1981; Smith et al., 1983). In the ovary, THC has been shown to inhibit follicular steroidogenesis both *in vivo* (Zoller, 1985) and *in vitro* (Burstein et al., 1979; Moon et al., 1982; Reich et al., 1982; Lewysohn et al.,

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1984). It is now known that similar to THC, the endocannabinoid anandamide also causes a decrease in serum LH, prolactin, and progesterone, as well as an increase in stillbirths in the pregnant rat (Wenger et al., 1997). One major signal transduction pathway for THC and the endocannabinoids is via the G-protein-coupled CB_1 and CB_2 receptors (Howlett and Mukhopadhyay, 2000). Over a decade ago, the CB_1 receptor mRNA has been shown to be present in the human ovary (Galiegue et al., 1995). Recently, the presence of both CB_1 and CB_2 receptors as well as anandamide and its biosynthetic enzyme *N*-acylphosphatidylethanolamine-phospholipase D and the degrading enzyme fatty acid amide hydrolase (FAAH) has been demonstrated in the human ovary (El-Talatini et al., 2009). An earlier study also demonstrated the presence of anandamide in the human follicular fluid (Schuel et al., 2002). Thus, potential for endocannabinoid signaling exists in the ovary. In fact, recent review articles have drawn attention to the importance of the endocannabinoid system in mammalian reproductive function (Taylor et al., 2007; Battista et al., 2008; Maccarrone, 2009). However, presently it is not known if any member of the endocannabinoid signaling system is present in the ovary of other mammals in addition to human. Therefore, we sought to determine if the G-protein-coupled cannabinoid receptors CB_1 and CB_2 and the anandamide-degrading enzyme FAAH are present in the rat ovary. Our data demonstrate that the components of the endocannabinoid signaling system are indeed expressed differentially in time and space in specific cell types of the rat ovary.

MATERIALS AND METHODS

Animal Model

All of the experiments described in this study conform to the guide for the care and use of laboratory animals, published by the National Research Council (Publications No. 0-309-05377-3, 1996) and were approved by the Kent State University Animal Care and Use Committee.

Twenty-two to twenty-five day old immature female Sprague-Dawley rats were injected subcutaneously with 15 international units (IU) of pregnant mare's serum gonadotropin (PMSG; Sigma-Aldrich, St. Louis, MO) in 100 μL of phosphate buffered saline (PBS) containing 0.2% (w/v) bovine serum albumin (BSA) or PBS alone (control). Two days later, PBS-treated rats and some of the PMSG-treated rates were sacrificed. The remaining PMSG-treated rats were injected with 5 IU of human chorionic gonadotropin (hCG) (Sigma-Aldrich, St. Louis, MO) in 100 μL of PBS-BSA. PMSG-treated preovulatory follicles ovulate in response to hCG and subsequently luteinize to form the corpora lutea of pseudopregnancy. The animals were sacrificed at three separate times after PMSG injection: (a) when preovulatory follicles are present (48 hr after PMSG), (b) 4, and (c) 14 days after hCG injection. The ovaries were removed and processed for immunofluorescent localization and Western analysis of CB_1 receptor and FAAH as described below. Observations were made from ovaries obtained from at least three animals at each time point. The cerebellum and liver were also removed for use as positive controls for CB_1 receptor and FAAH, respectively, in Western analysis. In addition, both FAAH wild-type and knockout mouse liver were obtained from Dr. Benjamin Cravatt of the Scripps Research Institute.

Tissue Processing and Immunoflurorescence Microscopy

The ovaries were frozen fresh in O.C.T. compound (Lab-Tek Products, Naperville, IL) and 8-μm thick sections were cut in a cryostat set at −15°C. Immunostaining was performed as before with minor modifications (Bagavandoss, 1998). Briefly, the sections were fixed in cold acetone for 10 min and air-dried. Subsequently, the sections were washed in PBS-0.2% BSA-0.05% Tween 20 (PBST) and incubated for 2 hr at 25°C or overnight at 4°C with following rabbit polyclonal antibodies in PBST ± blocking peptides: CB_2 antibody prepared against residues 20–33 of human CB_2 receptor (Cayman Chemicals, Ann Arbor, MI) at 5 μg/mL; CB_1 antibody prepared against residues 400–460 of the human CB_1 receptor (Dr. Ken Mackie, Indiana University, Bloomington) at 1:200 dilution; FAAH antibody prepared against residues 33–579 of rat FAAH enzyme (Dr. Benjamin Cravatt, Scripps Research Institute, San Diego) at 1:250 dilution. After three 5-min washes in PBST, the sections were incubated with fluorescein isothiocyanate (FITC) or rhodamine isothiocyanate (TRITC)-conjugated secondary antibodies (Organon Teknika Corporation, Durham, NC) for 30–40 minutes at 25°C, washed and mounted in Vectashield® mounting medium (Vector laboratories, Burlingame, CA).

For double labeling, sections were simultaneously incubated with FAAH or cannabinoid receptor antibody and a guinea pig polyclonal antibody (Reed et al., 1993) prepared against rat type I interstitial collagen at 1:50 dilution. Subsequently, the sections were washed as above and incubated simultaneously with FITC-goat anti-rabbit and TRITC goat anti-guinea pig secondary antibodies, rinsed in PBST and mounted as above. The sections were viewed and photographed using an Olympus inverted microscope equipped with Olympus Fluo-View, three-laser confocal microscopy system.

Western Analysis

The PMSG-primed ovaries, liver, and the cerebellum were homogenized in buffer (Plet et al., 1982) containing tris(hydroxymethyl) aminomethane hydrochloride (Tris-HCl 20 mM, pH 7.5), sucrose (0.25 M), $MgCl_2$ (2.5 mM), EDTA (2.5 mM), KCl (10 mM), thimerosal (0.02%), and a protease inhibitor cocktail (Sigma-Aldrich, MO). The homogenate was centrifuged for 10 min at 800 g; the supernatant was collected and centrifuged again at 20,000 g for 20 min. Oocytes were isolated from PMSG-injected rat ovaries (Mehlmann and Kline, 1994) and dissolved in 0.25% octylglucoside containing protease inhibitors. Equal amounts of reduced proteins (~200 μg/lane) were separated on 10% SDS-polyacrylamide gel. The proteins were then transferred to a polyvinylidene fluoride membrane and blocked overnight at 4°C in PBS containing nonfat dry milk (5%), Tween-20 (0.05%), and magnesium chloride (2.5 mM). The blots were incubated overnight at 4°C with an affinity purified rabbit polyclonal antibody (1:300 dilution) prepared against GST fusion protein containing the first 77 amino terminal residues (GST-CB_1:1–77) of the CB_1 receptor (Twitchell et al., 1997).

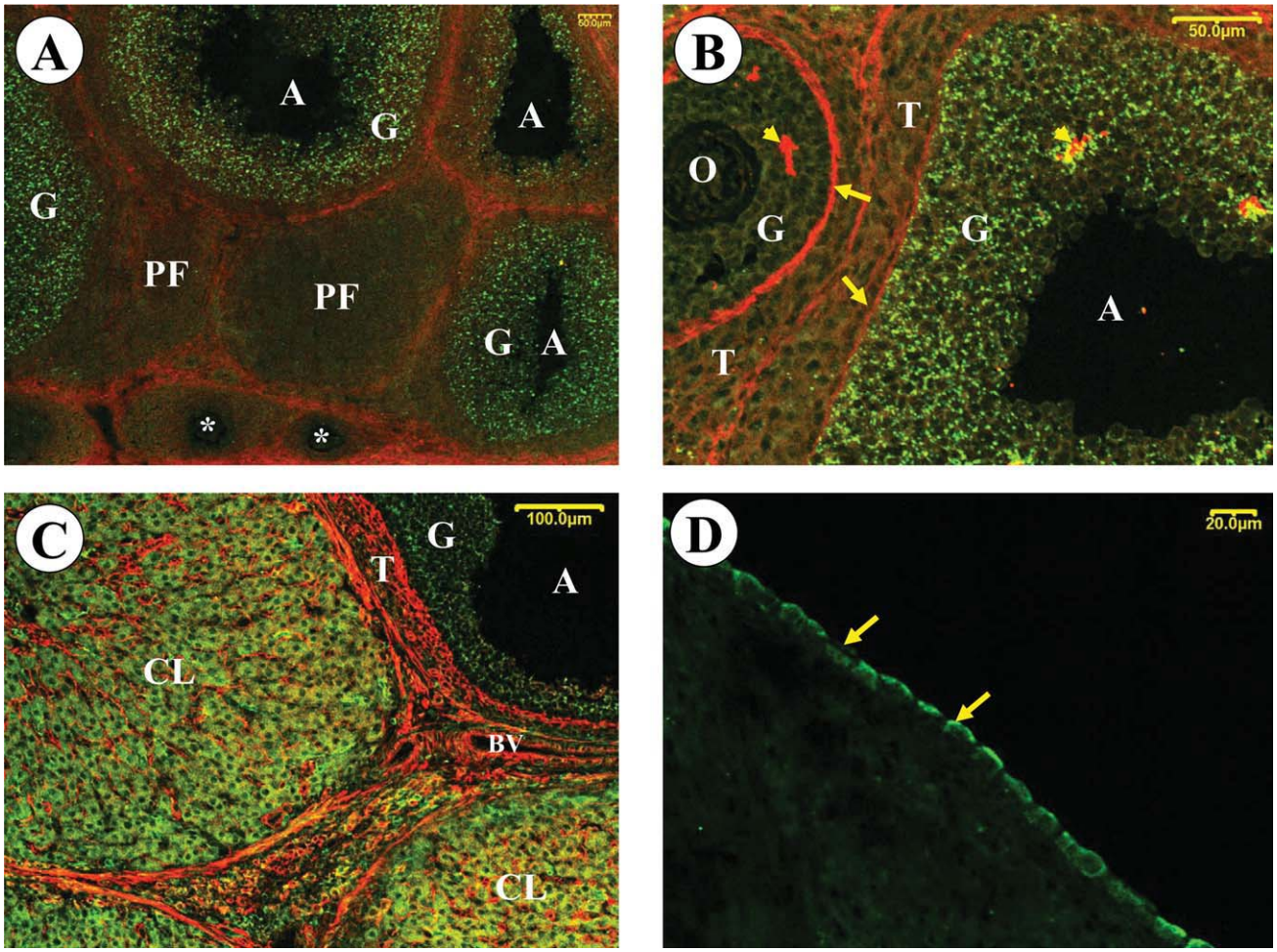

Fig. 1. (**A**). Panoramic view of a PMSG-injected ovary showing the presence of CB_1 receptors in granulosa cells of antral follicles. Note the absence of CB_1 staining in preantral follicles (PF) and the oocytes (*). The section was doubled labeled with antibodies to both CB_1 receptor (green) and type I interstitial collagen (red). (**B**) A close-up view of two follicles from a PMSG-injected rat ovary: The reticular layer of the basal lamina is clearly outlined by the collagen antibody (arrows). Note that the CB_1 receptor immunoreactivity is present only in granulosa cells (G) of the large antral follicle on the right. The oocyte (O), theca (T), and the granulosa cells (G) of a small follicle do not exhibit CB_1 receptor immunoreactivity. Fragments of the collagen from sectioning artifact can also be seen within the follicles (arrowheads). (**C**) Ovary from a day 4 pseudopregnant rat: The luteal cells of the corpora lutea (CL) show immunoreactivity to the CB_1 receptor antibody. Unlike in the granulosa cells, the staining is smooth rather than punctate, which is likely because of the large cytoplasmic to nuclear ratio of the luteal cells. Granulosa cells (G) in an antral follicle also bind to the CB_1 receptor antibody. The red counterstaining throughout the section illustrates the presence of interstitial collagen in the ovary. (**D**) A section from a PBS-injected rat ovary shows the presence of CB_1 receptor in the OSE (arrows). A = antrum; BV = blood vessel; CL = corpus luteum; G = granulosa cells; O = oocyte; T = theca.

Western blot against FAAH was performed with a rabbit polyclonal antibody raised against rat residues 561–579 of FAAH (Cayman Chemicals, Ann Arbor) at 1:250 dilution. The membrane was washed in PBST and the bound antibody was probed with an enhanced chemiluminescent detection kit (Amersham Biosciences, NJ). Control blots were incubated with the primary antibody, which was preincubated with 2 μg of the blocking peptide for 1 hr at room temperature.

RESULTS

Distribution of CB_1 Receptor

The CB_1 receptor distinctly localizes to the plasma membrane of granulosa cells (Fig. 1A, B). Although the receptors are present in the granulosa cells of antral follicles, none is present in the granulosa cells of the preantral follicles (Fig. 1A, B). CB_1 receptor is also not present in oocytes or thecal cells (Fig. 1A, B). In 4-day old corpus luteum (CL), which secretes increasing concentrations of progesterone, CB_1 receptor is expressed by the luteal cells (Fig. 1C). During this time, the granulosa cells of the follicles continue to show staining for the CB_1 receptor (Fig. 1C). The ovarian surface epithelium (OSE) is also immunoreactive to the CB_1 receptor antibody (Fig. 1D). The red counterstaining with interstitial collagen antibody shows the wide distribution of this collagen throughout the ovary (Fig. 1A–C). Note the distinct localization of this collagen in the reticular layer (Bagavandoss et al., 1983) of the basal lamina (Fig. 1B, arrows).

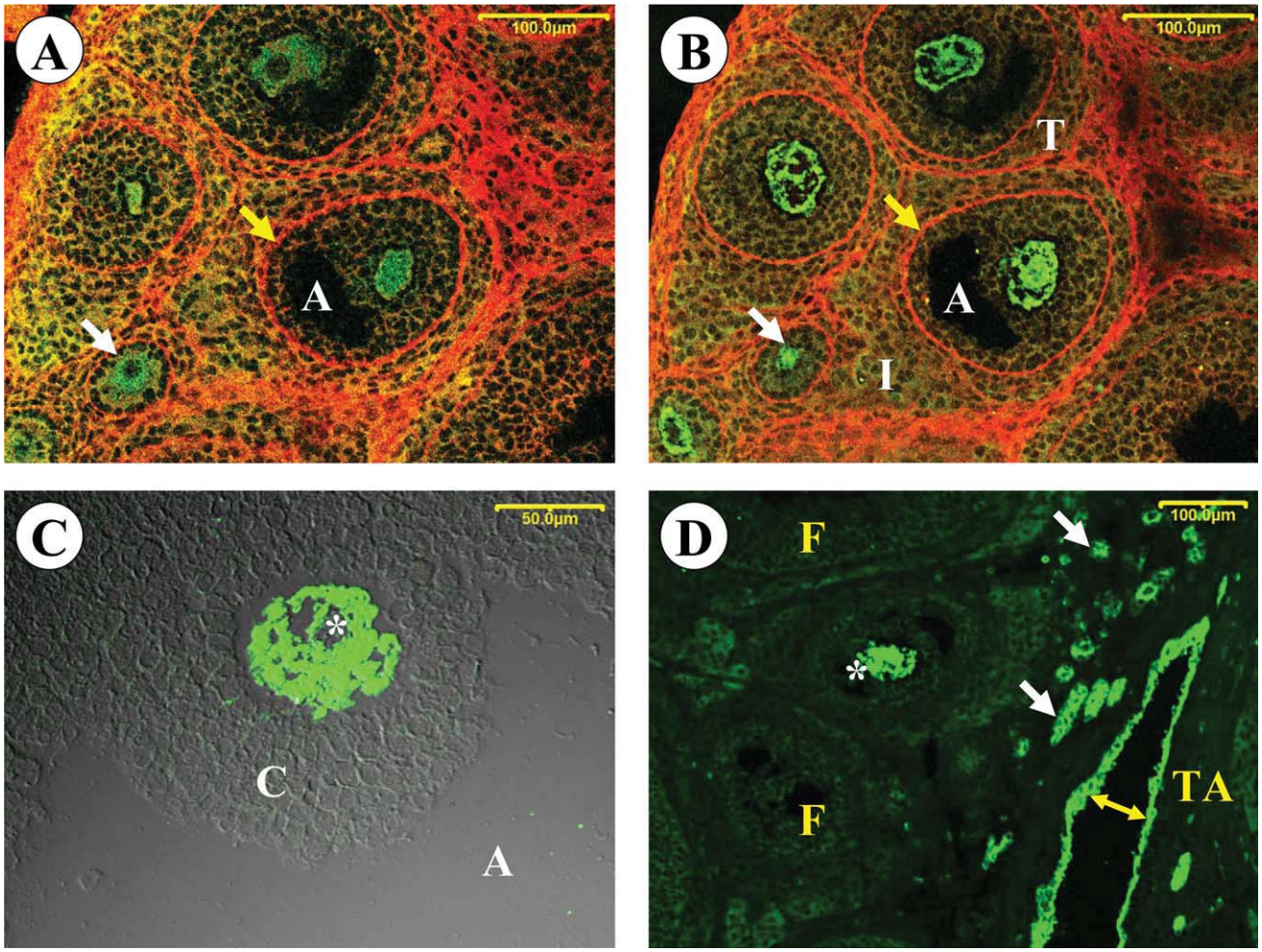

Fig. 2. PMSG-injected ovary shows the presence of CB_2 and FAAH in adjacent sections in green fluorescence (**A**, **B**). Both sections are also double-labeled with anti-rat type I collagen (red fluorescence). Note that the same oocyte shows immunoreactivity to both FAAH (A) and CB_2 (B). Even the oocyte in a primordial follicle shows FAAH and CB_2 staining (white arrows). The follicular basement membrane is clearly outlined by the type I collagen antibody (yellow arrows). A composite immunofluorescent and differential interference contrast image of a large antral follicle (**C**) shows staining for FAAH only in the oocyte. Surrounding cumulus cells (C) show no staining for FAAH. In panel **D**, OSE lining the ovarian crypt shows FAAH staining (yellow double arrows). Also note the presence of FAAH in the epithelial cords (white arrow) of the tunica albuginea (TA) and in individual cells in adjacent areas (white arrows). A = antrum; F = follicle; * = oocyte; TA= tunica albuginea.

Distribution of CB_2 and FAAH

Unlike the CB_1 receptor, CB_2 receptor is not present in the granulosa cells of the follicles. CB_2 receptor is expressed exclusively by the oocytes of both preantral and antral follicles (Fig. 2A). FAAH antibody also stains the oocytes in the follicles (Fig. 2B, C). Even in the preovulatory follicles FAAH localization remains confined to the oocyte without any reactivity in the surrounding cumulus cells (Fig. 2C). Similar to CB_2, thecal and granulosa cells do not show any immunoreactivity for FAAH antibody. However, strong FAAH immunoreactivity is present in the OSE (Fig. 2D, 3A) and in individual cells and subepithelial cords of the tunica albuginea (TA) below the OSE (Fig. 2D, 3A). Many FAAH-positive cells are scattered throughout the cortex below the TA and adjacent to developing preantral follicles (Fig. 3A, B).

In day 4 progesterone secreting CL, the luteal cells stain strongly for both FAAH (Fig. 4A) and CB_2 (Fig. 4B). FAAH staining is also observed in the interstitium (Fig 4A). By 14th day of pseudopregnancy, when the CL is undergoing regression, staining for both FAAH (Fig 4C) and CB_2 (Fig. 4D) are drastically reduced in the luteal cells. However, the interstitial cells and the oocytes continue to remain positive for both FAAH (Fig. 4C) and CB_2 (Fig. 4D), respectively.

Western blot of CB_1 and FAAH

A M_r 60 kDa CB_1 receptor was present both in the PMSG primed ovary and in the cerebellum (Fig. 5A), which served as a positive control (Tsou et al., 1998). The binding is specific as preabsorption of the antibody

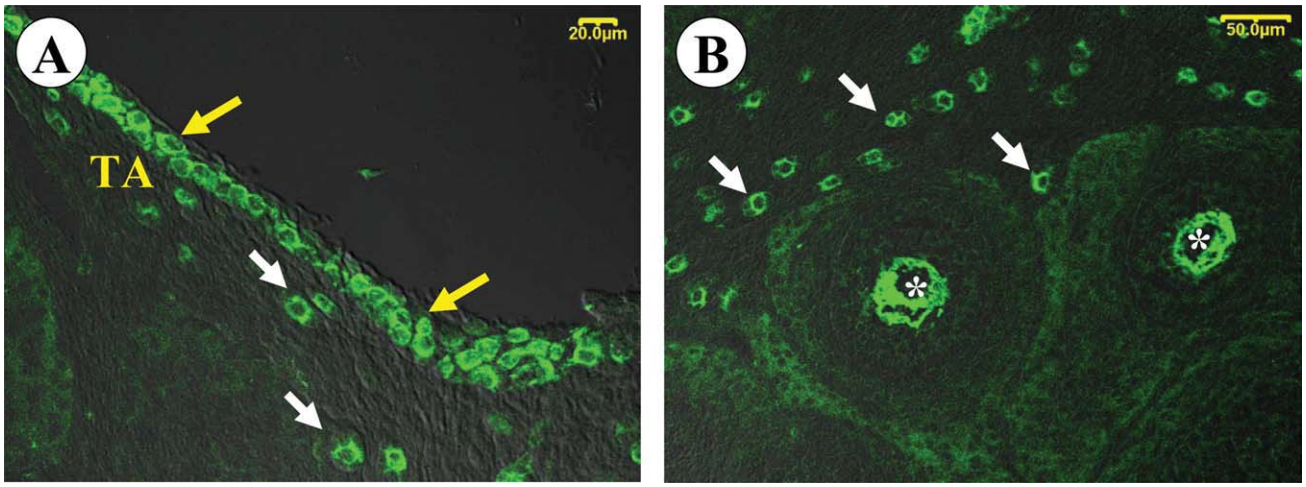

Fig. 3. FAAH staining in a ovary from another rat. **A**. FAAH in OSE (yellow arrows). Individual cells staining for FAAH in the tunica albuginea (TA) and below it (white arrows). **B**. Many single cells stain for FAAH in the ovarian cortex adjacent to the preantral follicles (white arrows). Also note the staining for FAAH in the oocytes of these follicles (*).

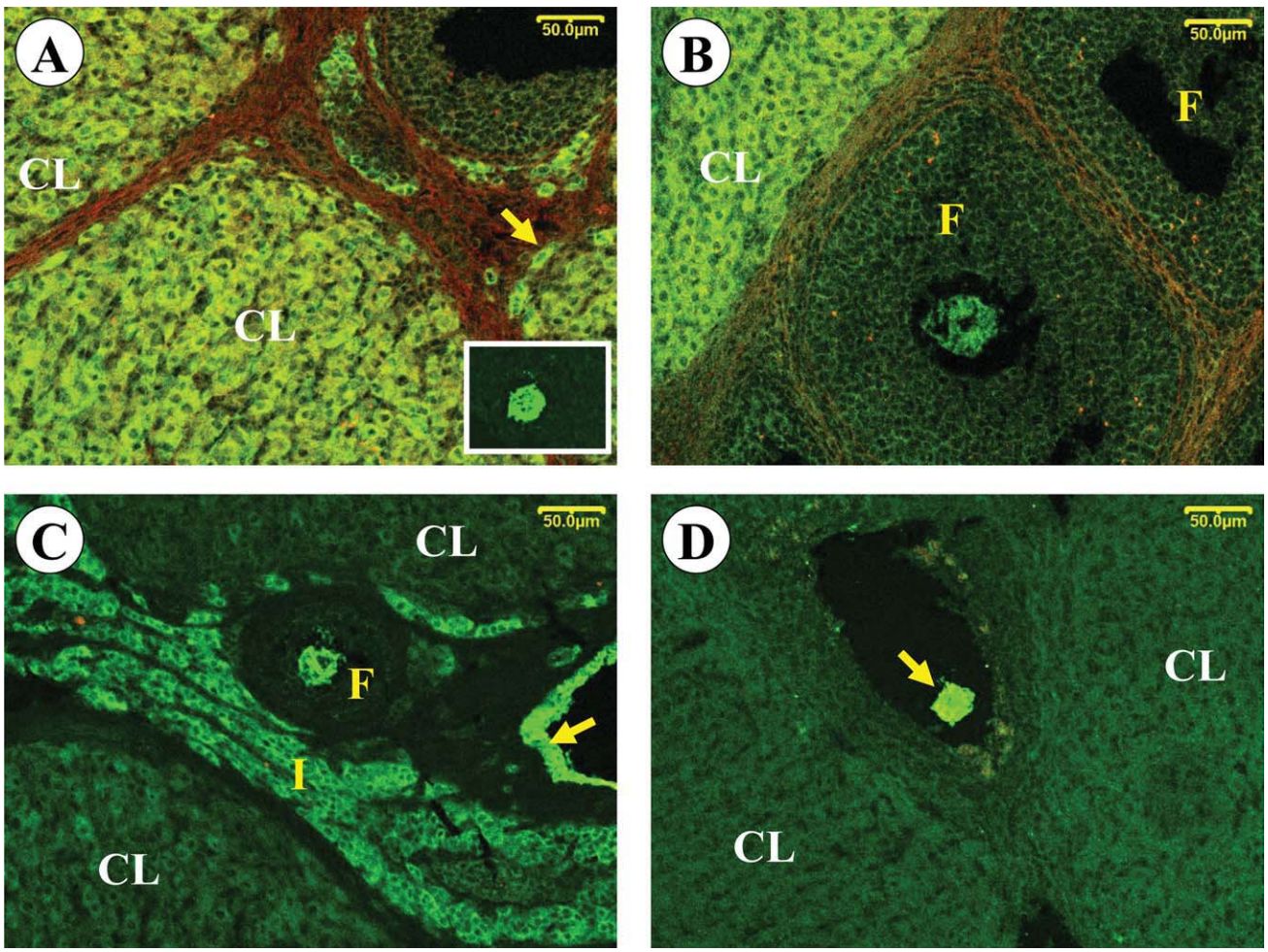

Fig. 4. Ovary from a day 4 pseudopregnant rat (**A**, **B**): The luteal cells of functional corpora lutea (CL) and a oocyte from a follicle (inset) stain for FAAH (A) and CB_2 (B). FAAH staining is also present in some interstitial cells (yellow arrow). Ovary from a day 14 pseudopregnant rat (**C**, **D**): Note the presence of FAAH (C) and CB_2 (D) immunoreactivity in the oocytes. The oocyte staining (D) for CB_2 appears to be in an atretic follicle (arrow). In the regressing corpora lutea (CL) at the end of pseudopregnancy, FAAH (C) and CB_2 (D) staining are essentially absent.

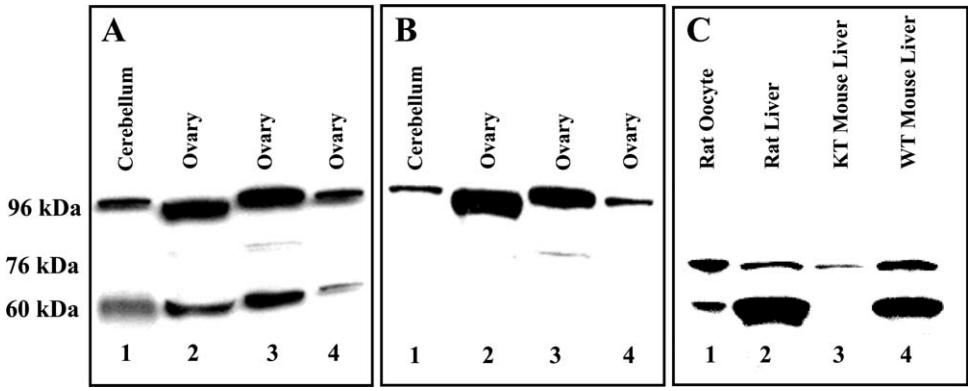

Fig. 5. Western blots of CB_1 (**A**, **B**) and FAAH (**C**). Membrane pellets from the cerebellum and ovaries of PMSG-injected rats (A, B) were subjected to electrophoresis and subsequent Western blotting as described in the methods. (A) The CB_1 antibody not only binds to the 60 kDa CB_1 receptor but also recognizes a 96 kDa protein. However, incubation of the membrane with the antibody preabsorbed with a blocking peptide (to AA 1-77) completely prevents its binding to the M_r 60 kDa band, whereas binding to the 96 kDa protein persists (B). Lanes 2 and 3 represent the membrane pellets from two separate experiments. Lane 4 represents the 20,000 g supernatant of the sample in lane 3. (C) Western blot of solubilized proteins from 108 oocytes and livers from a rat, FAAH knockout (KT), and wild-type (WT) mice. A 60 kDa FAAH was detected in the rat oocytes, rat liver and the WT mouse liver. As expected, the 60 kDa protein was not detected in the FAAH KT mouse liver. An additional nonspecific 76 kDa protein was also detected, which was present in all samples; including the FAAH KT mouse liver.

with the blocking peptide selectively abolished the M_r 60 kDa CB_1 band (Fig. 5B). The antibody also binds to a M_r 96 kDa protein, which is not blocked by the blocking peptide (Fig. 5A, B). Western analyses of FAAH in the oocytes show that the antibody, in addition to staining the expected M_r 60 kDa FAAH protein in both the rat and wild-type mouse livers (positive control), stains a nonspecific M_r 76 kDa protein (Fig. 5C). Specificity of the antibody is shown by the absence of M_r 60 kDa FAAH staining in the FAAH knockout mouse liver (Fig. 5C).

DISCUSSION

Using an established immature rat model for follicular development and CL formation (Bagavandoss, 1998), we have studied the presence of cannabinoid receptors CB_1 and CB_2 as well as FAAH, the major enzyme that regulates the concentration of the endocannabinoid anandamide in cells (Mckinney and Cravatt, 2005). In this model, within 48 hours after PMSG injection, many follicles develop into preovulatory follicles. Subsequently, in response to hCG, pseudopregnancy begins as the follicles ovulate and transform into CL. By fourth day of pseudopregnancy, the CL is highly steroidogenic and secretes increasing amount of progesterone (Horikoshi and Wiest, 1971). By 14th day, however, the ephemeral CL predictably shows marked decline in progesterone secretion and undergoes structural involution and regression. Therefore, this animal model is well suited for studying the distribution of biomolecules during follicular and luteal phases of the ovary.

The results of our study demonstrate the differential expression of both cannabinoid receptors and FAAH during different stages of ovarian function. During follicular development, CB_1 receptor is present only in the antral follicles of granulosa cells. It is known that though the development of preantral follicles is not dependent upon gonadotropins, subsequent antral development requires FSH and estradiol (Robker and Richards, 1998 and references therein). Therefore, the appearance of CB_1 receptor in the antral follicles suggests that these receptors are induced in response to FSH-like activity of PMSG.

Unlike the CB_1 receptor, CB_2 receptor is not present in the granulosa cells during any stage of follicular development. Furthermore, CB_2 receptor is restricted to the oocytes of both preantral and antral follicles. The differential distribution of CB_1 and CB_2 receptors in the follicle suggests that during follicular development ovarian anandamide (Schuel et al., 2002, El-Talatini et al., 2009) will simultaneously act on two distinct cell types through two distinct cannabinoid receptors. During pseudopregnancy, both cannabinoid receptors are concurrently present in the functional luteal cells but not in the cells of regressing CL.

Recently, during the preparation of this manuscript, both CB_1 and CB_2 receptors have been demonstrated in the adult human ovary (El-Talatini et al., 2009). Here too, CB_1 receptor is expressed primarily in the large antral follicles and the functioning CL with less intense expression in small follicles and corpus albicans. However, some expression was also observed in the oocyte and theca. Similar to our study, CB_2 receptor was observed in the oocytes of both preantral and antral follicles. However, unlike in our study, granulosa cells of the human follicles were also shown to express CB_2 receptor (El-Talatini et al., 2009). In our study, we have used immunofluorescence, whereas the study on human ovaries was conducted with biotinylated secondary antibody and ABC Elite Reagent (El-Talatini et al., 2009). Therefore, it is not clear if these observed differences reflect from differences between species or differences in methodology.

Both CB_1 (Devane et al., 1988; Matsuda et al., 1990) and CB_2 receptors (Munro et al., 1993) are G-protein-coupled receptors. THC and the endocannabinoids anandamide and 2-AG activate both these receptors (Howlett

and Mukhopadhyay, 2000 and references therein). Activation of these receptors results in multiple signal transduction mechanisms, including the inhibition of adenylyl cyclase and consequent decrease in cAMP (Matsuda et al., 1990; Felder et al., 1992; Vogel et al., 1993) or stimulation of adenylyl cyclase and corresponding increase in cAMP (Glass and Felder, 1997; Rodriguez de Fonseca et al., 1999). In fact, in rat granulosa cell cultures, THC has been shown to inhibit LH-stimulated cAMP and progesterone production (Lewysohn et al., 1984). Cannabinoids also inhibit both basal and FSH-stimulated progesterone production in granulosa cells (Moon et al., 1982). In rat luteal cells too cannabinoids have been shown to inhibit steroidogenesis (Burstein et al., 1979). Thus, in granulosa and luteal cells potential exists for cross talk between signal transduction pathways generated by gonadotropins and endocannbinoids.

FAAH regulates the concentration of anandamide by metabolizing it into arachidonic acid and ethanolamide (McKinney and Cravatt, 2005). Both FAAH and CB_2 receptor are concurrently present in the oocytes of preantral and antral follicles. The consequences of such presence are two-fold: One, by decreasing the concentration of anandamide, FAAH could potentially regulate the availability of anandamide to the CB_2 receptor. Two, FAAH-generated arachidonic acid itself could serve as a signal or a substrate for additional cellular signaling in follicles, including the oocyte. In fact, arachidonic acid has been shown to be a constituent of mammalian oocytes (Homa et al., 1986; Matorras et al., 1998; McEvoy et al., 2000; Kim et al., 2001). Further, the presence of FAAH in the oocyte invokes additional possibilities for follicular maturation. For example, oocyte maturation and cumulus expansion require both cyclooxygenase 2 (COX-2) and prostaglandin E2 (Takahashi et al., 2006). However, COX-2 is expressed only by the cumulus cells not the oocyte (Dell'Aquila et al., 2004; Takahashi et al., 2006; Feuerstein et al., 2007). Thus, FAAH-generated arachidonic acid from the oocyte could serve as a major substrate for COX-2 in the neighboring cumulus cells for the production of prostaglandin. The presence of both FAAH and CB receptors in the CL also suggests the existence of regulated cannabinoid-mediated signaling in the luteal cells. For example, arachidonic acid generated from the luteal cell FAAH could affect the production of progesterone by the CL as arachidonic acid itself has been shown to stimulate progesterone production in the rat luteal cells (Wang and Leung, 1988).

The presence of FAAH and CB_1 receptor in the OSE is quite intriguing. As the OSE is a major source of ovarian cancer, potential exists for cannabinoid signaling in ovarian carcinogenesis. FAAH was also present in the OSE-associated crypts, subepithelial cords of the tunica albuginea, and in the cells scattered in the ovarian cortex. It is not clear if these FAAH positive cells in the ovarian cortex migrate from the OSE or from the subepithelial cords and contribute to any cells during follicular development. Results from human, sheep, and mouse suggest such a possibility. Data from an elegant study in the adult human ovary indicate that the OSE and the subepithelial cords in the TA constitute a dynamic population of cells capable of differentiating into presumptive granulosa or germ cells (Bukovsky et al., 2004). In the fetal sheep ovary, it has been recently suggested that greater than 95% of the granulosa cells in the newly formed primordial follicles originate from the OSE (Sawyer et al., 2002). In the adult mouse ovary at least some of the cells in OSE appear to be the source of stem cells (Johnson et al., 2004). If OSE does contribute to other parenchymal cells of the rat ovary, we suggest that FAAH might serve as a potential marker for tracking the cells of the OSE. In summary, results of our study show both the presence and differential distribution of CB_1 and CB_2 cannabinoid receptors and the anandamide-metabolizing enzyme FAAH in the rat ovary. Therefore, potential exists for the regulation of ovarian physiology by the endocannabinoid system.

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