EP3 receptor isoforms are differentially expressed in subpopulations of primate granulosa cells and couple to unique G-proteins

Soon Ok Kim¹, Brandy L Dozier^{1,†}, Julie A Kerry² and Diane M Duffy¹

Departments of ¹Physiological Sciences and ²Microbiology and Molecular Cell Biology, Eastern Virginia Medical School, PO Box 1980, Norfolk, Virginia 23501, USA

Correspondence should be addressed to D M Duffy; Email: duffydm@evms.edu

[†]B L Dozier is now at the Oregon National Primate Research Center, Beaverton, Oregon 97006, USA

Abstract

Prostaglandin E₂ (PGE₂) produced within the ovarian follicle is necessary for ovulation. PGE₂ is recognized by four distinct G-protein-coupled receptors. Among them, PTGER3 (also known as EP3) is unique in that mRNA splicing generates multiple isoforms. Each isoform has a distinct amino acid composition in the C-terminal region, which is involved in G-protein coupling. To determine whether monkey EP3 isoforms couple to different G-proteins, each EP3 isoform was expressed in Chinese hamster ovary cells, and intracellular signals were examined after stimulation with the EP3 agonist sulprostone. Stimulation of EP3 isoform 5 (EP3-5) reduced cAMP in a pertussis toxin (PTX)-sensitive manner, indicating involvement of G α i. Stimulation of EP3-9 increased cAMP, which was reduced by the general G-protein inhibitor GDP- β -S, and also increased intracellular calcium, which was reduced by PTX and GDP- β -S. So, EP3-9 likely couples to both G α s and a PTX-sensitive G-protein to regulate intracellular signals. Stimulation of EP3-14 increased cAMP, which was further increased by PTX, so EP3-14 likely regulates cAMP via multiple G-proteins. Granulosa cell expression of all EP3 isoforms increased in response to an ovulatory dose of human chorionic gonadotropin. Two EP3 isoforms were differentially expressed in functional subpopulations of granulosa cells. EP3-5 was low in granulosa cells at the follicle apex while EP3-9 was high in cumulus granulosa cells. Differential expression of EP3 isoforms may yield different intracellular responses to PGE₂ in granulosa cell subpopulations, contributing to the different roles played by granulosa cell subpopulations in the process of ovulation.

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Introduction

Prostaglandin (PG) production by the follicle is an essential prerequisite for successful ovulation (Murdoch *et al.* 1993). The midcycle surge of luteinizing hormone (LH) stimulates PG production by granulosa cells of ovulatory follicles, elevating follicular PGs to peak levels just before ovulation (Wong & Richards 1991, Liu *et al.* 1997, Sirois & Doré 1997, Duffy & Stouffer 2001). Among PGs, PGE₂ has been identified as the key PG that regulates essential ovulatory events including cumulus expansion, follicle rupture, and oocyte release. Inhibition of PG synthesis *in vivo* blocks ovulation, whereas co-treatment with PGE₂ restores ovulation, supporting a crucial role for PGE₂ in ovulation (Tsafiri *et al.* 1972, Duffy & Stouffer 2002, Peters *et al.* 2004).

PGE₂ exerts its actions by binding to four distinct G-protein-coupled receptors (GPCRs): PTGER1, PTGER2, PTGER3, and PTGER4 (also known as EP1, EP2, EP3, and EP4 respectively; Coleman *et al.* 1994, Narumiya *et al.* 1999). Among EP receptors, EP3 is

unique in that alternative mRNA splicing gives rise to multiple isoforms. All EP3 isoforms share a common N-terminal sequence, which includes hormone binding and membrane spanning regions. However, each isoform has a unique amino acid composition in the C-terminal region, which regulates intracellular location and plays a key role in G-protein coupling.

EP3 receptor-mediated intracellular signal transduction has been studied in many tissues. EP3 receptors have often been shown to inhibit cAMP generation by reducing adenylyl cyclase activity via G α i. EP3 receptors have also been shown to activate phospholipase C to release intracellular calcium via G α q (Yang *et al.* 1994, Schmid *et al.* 1995). An unknown pertussis toxin (PTX)-sensitive G-protein can also link EP3 to regulation of intracellular calcium (Tomić *et al.* 2002). Less frequently, EP3 has been shown to increase adenylyl cyclase activity by coupling to G α s or to activate the small G-protein Rho by coupling to G_{12/13} (An *et al.* 1994, Tamma *et al.* 2003).

EP3 receptors have been implicated in ovulatory events in large animal species, including primates. EP3 receptors are expressed in mural and cumulus granulosa cells of ovarian follicles, with increased expression after the ovulatory gonadotropin surge (Tsai *et al.* 1996, Calder *et al.* 2001, Markosyan *et al.* 2006, Bridges & Fortune 2007, Harris *et al.* 2011). High EP3 expression in bovine cumulus cells correlates with improved quality of the oocyte and the surrounding cumulus (Calder *et al.* 2001). A role for EP3 receptors to promote luteinization, an essential event in ovulatory cascade of large animal species, has also been suggested, and EP3 mRNA is highly expressed in bovine and monkey luteal cells (Tsai *et al.* 1996, Bogan *et al.* 2008a, 2008b). In monkey granulosa cells, EP3 receptors regulate both tissue-type plasminogen activator (PLAT) and plasminogen activator inhibitor type 1 (SERPINE1), key mediators of proteolysis associated with follicle rupture (Markosyan & Duffy 2009). While mice lacking EP3 expression exhibit no gross reproductive abnormalities (Fleming *et al.* 1998), EP3 receptors have been implicated in essential ovulatory events in large animal species, which ovulate a single follicle.

The objectives of this study were to i) determine which EP3 receptor isoforms are expressed in monkey granulosa cells during the ovulatory interval, ii) identify the intracellular signals regulated by each monkey EP3 isoform, and iii) examine the distribution of EP3 isoforms among subpopulations of granulosa cells within the primate ovulatory follicle. Differential expression of EP3 isoforms may allow different roles for each granulosa cell subpopulations in the overall process of ovulation in response to ovulatory concentrations of PGE₂.

Materials and methods

Animal protocols

Granulosa cells and whole ovaries were obtained from adult female cynomolgus macaques (*Macaca fascicularis*, aged 4–10 years, 2.5–4.0 kg) at Eastern Virginia Medical School (Norfolk, VA, USA). All animal protocols and experiments were approved by the Eastern Virginia Medical School Animal Care and Use Committee and were conducted in accordance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals. Animal husbandry and sample collections were performed as described previously (Duffy *et al.* 2005a). Briefly, adult females were checked regularly for menstruation, and the first day of menstruation was designated as day 1 of the menstrual cycle. Blood samples were obtained following ketamine chemical restraint (5–10 mg/kg body weight; Henry Schein, Port Washington, NY, USA) by femoral venipuncture, and serum was stored at –20 °C. Aseptic surgeries were performed in a dedicated surgical suite under isoflurane anesthesia using midline laparotomy or laparoscopic procedures.

A controlled ovarian stimulation model, developed for the collection of multiple oocytes for IVF, was used to obtain

monkey granulosa cells and whole ovaries (Vandevoort *et al.* 1989). Beginning within 3 days of initiation of menstruation, recombinant human follicle-stimulating hormone (r-hFSH; 90 IU daily; Merck & Co.) was administered for 6–8 days, followed by daily administration of 90 IU r-hFSH plus 60 IU r-hLH (Serono Reproductive Biology Institute, Rockland, MA, USA) for 2–3 days to stimulate the growth of multiple preovulatory follicles. The gonadotropin-releasing hormone antagonist Ganirelix acetate (30 µg/kg per day; Merck & Co.) or Antide (0.5 mg/kg per day; Serono) was also administered daily to prevent an endogenous ovulatory LH surge. Adequate follicular development was monitored by ultrasonography and serum estradiol and progesterone levels using the Immulite 1000 immunoassay system (Siemens Medical Diagnostics Solutions, Flanders, NJ, USA). Follicular aspiration was performed before (0 h), 12, 24, and 36 h after administration of 1000 IU recombinant human chorionic gonadotropin (hCG; Serono) using a 22 gauge syringe to collect the aspirates of all follicles with a diameter of more than 4 mm. Whole ovaries were also removed from additional monkeys undergoing controlled ovarian stimulation before and after hCG administration. These times were selected to span the monkey ovulatory interval. Ovulation typically occurs about 40 h after the endogenous LH surge in natural menstrual cycles (Weick *et al.* 1973). Consistent with our previous studies, ovulation was not observed before or at 36 h following hCG administration.

To obtain granulosa cells, follicular aspirates were centrifuged at 250 g. Pelleted cells were resuspended, and after oocyte removal, a granulosa cell-enriched population of the remaining cells was obtained by Percoll gradient centrifugation (Chaffin *et al.* 1999). Viability of granulosa cell-enriched preparations was assessed by trypan blue (Sigma–Aldrich) exclusion and averaged 85%.

3'-RACE and cloning

Total RNA was isolated from monkey granulosa cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions with the addition of 25 µg/ml glycogen (Roche Diagnostics) at the RNA precipitation step. The full-length cDNA sequences of EP3 receptor variable regions were obtained using the RACE Kit (Roche). Briefly, total RNA obtained from granulosa cells was reverse transcribed using an oligo-dT anchor primer. cDNA was amplified by PCR with a PCR anchor and a gene-specific forward primer for the EP3 common region (5'-TTATCAGGAGCGGAGAC-3') previously designed in our laboratory (Markosyan *et al.* 2006). To obtain the full-length cDNA sequence of the EP3 common region, conventional PCR was performed using a forward primer 5'-GTAAACGCCGACCTCC-3' and a reverse primer 5'-GCAAACTTTCTGAAGAAGG-3'. The RACE fragments of the EP3 variable regions and the EP3 common region cDNA were gel purified using the Qiaquick gel extraction kit (Qiagen) and cloned using the StrataClone PCR Cloning Kit (Agilent Technologies, Wilmington, DE, USA) for amplification and sequencing. For the StrataClone PCR cloning system, forward primer 5'-CGGGACTAGCTCTTCG-3' and reverse primer 5'-TTATCAGGAGCGGAGAC-3' for variable regions and forward primer 5'-GCTCTCTGGACGCTAT-3' and reverse primer

5'-GCAAACTTTCGAAGAAGG-3' for EP3 common region were used. In order to express each full-length EP3 receptor isoform, the EP3 common region and a single variable region were then ligated and subcloned into eukaryotic expression vectors. pcDNA3.1 (Invitrogen) has a powerful cytomegalovirus immediate early promoter and is widely used for mammalian transient expression. pSI (Promega) was also used as it has a weak simian virus 40 (SV40) promoter and results in reduced levels of expression of the inserted genes (Garmory *et al.* 2003). The correct open-reading frame and amino acid sequence for each EP3 receptor C-terminal region was determined using EMBOSS Transeq (EMBL-EBI, Hinxton, Cambridgeshire, UK).

Cell line culture and transfection

Chinese hamster ovary (CHO) cells (ATCC, Manassas, VA, USA) were cultured in F-12K (Kaighn's Modification of Ham's F-12) medium (ATCC) supplemented with 10% fetal bovine serum (Bench Mark, Millwood, WV, USA) and 1% antibiotic-antimycotic solution (Gibco) containing 10 000 µg/ml streptomycin, 10 000 units/ml penicillin, and 25 µg/ml amphotericin B. Cells were transfected by plasmid DNA expressing green fluorescent protein (GFP) or each EP3 receptor isoform using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Briefly, 1 day before transfection, cells were seeded in F-12K with no antibiotics and incubated until ~80% confluency. Cells were washed with PBS and cultured with the transfection complexes containing plasmid DNA in fresh medium with no supplements. Approximately 48 h after transfection, GFP expression was examined for transfection efficiency (80–90%), and cells were then used for each experiment. Mock-transfected cells supplemented with transfecting reagent alone served as a control. Cells transfected with empty vector, which was not carrying any EP3 receptor isoform, also served as a control.

Preliminary experiments (not shown) confirmed that CHO cells do not express endogenous EP3 receptors. EP3 receptors were not detected in mock-transfected or empty vector-transfected CHO cells by immunocytochemistry using an EP3 antibody, following methods previously described by this laboratory (Markosyan *et al.* 2006). Mock-transfected and empty vector-transfected CHO cells did not respond to PGE₂ or sulprostone with alterations in intracellular calcium or cAMP.

Immunofluorescence detection

The hemagglutinin (HA) tag was inserted before the EP3 common region of each isoform using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). Approximately 48 h after transfection, CHO cells were fixed with 10% buffered formalin (Fisher Diagnostics, Middletown, VA, USA) and blocked with 5% non-immune goat serum (Vector Labs, Burlingame, CA, USA) in PBS containing 0.1% Triton X (PBS-Triton) for 1 h at room temperature. Cells were incubated overnight with an anti-HA tag antibody (1:1000; Covance, Berkeley, CA, USA) followed by an Alexa Fluor 488-conjugated anti-mouse secondary antibody (400 µg/ml; Molecular Probes, Eugene, OR, USA). Finally, cells were

washed with PBS and mounted in Vectashield medium containing propidium iodide (Vector Labs) for nuclei staining. Images were obtained using a Zeiss LSM 510 confocal microscope (Carl Zeiss, Inc., Thornwood, NY, USA) at 488 nm (green) for EP3 receptor isoforms and 543 nm (red) for nuclei. Incubation with no primary antibody was used to determine background levels of fluorescence.

cAMP concentration

Transfected CHO cells were treated with the EP3 agonist sulprostone (1 µM; Cayman Chemical, Ann Arbor, MI, USA) or vehicle (0.01% DMSO) for 4 h. To block G-protein activity, some cells were preincubated with PTX (100 ng/ml; Sigma; 20 h) or GDP-β-S (100 µM; Sigma; 30 min) prior to treatment with sulprostone or vehicle. PTX is primarily known as a Gαi inhibitor but also inhibits one or more unidentified G-proteins that regulate intracellular calcium. GDP-β-S is a general G-protein inhibitor. Media were then assayed for cAMP levels by EIA (Cayman Chemical) following manufacturer's instructions including cAMP acetylation for maximum sensitivity. All samples were assayed in duplicate. cAMP levels were normalized to cell number of each well. Levels of cAMP in treated culture wells are expressed relative to cAMP in vehicle-only (control) wells. A single batch of transfected CHO cells was used for each independent experiment, providing data suitable for repeated measures analysis.

Calcium measurements

Assessments of intracellular calcium were performed using the Fluo-4 NW Calcium Assay Kit (Molecular Probes). Approximately 48 h after transfection, media were replaced with Fluo-4 NW dye mix and 2.5 mM probenecid diluted in Hanks' balanced salt solution with 20 mM HEPES. Cells were incubated at 37 °C for 30 min and then at room temperature for an additional 30 min. After determination of basal fluorescence before treatment (0 s), cells were stimulated with sulprostone (1 µM) or vehicle (0.01% DMSO). Some cells were preincubated with PTX (100 ng/ml; 20 h) or GDP-β-S (100 µM; 30 min) before sulprostone administration. Fluorescence was measured every 20 s for 2 min in a SpectraMax multimode microplate reader (Molecular Devices, Sunnyvale, CA, USA) with excitation at 494 nm and emission at 516 nm. Finally, cells were exposed to the calcium ionophore A23187 (10 µM; Calbiochem, San Diego, CA, USA) to confirm cell responsiveness. Fluorescence measured after sulprostone or A23187 treatment is expressed relative to basal level, where basal level is set equal to 1.0.

Real-time PCR

Total RNA isolated from monkey granulosa cells was reverse transcribed as described previously (Duffy *et al.* 2005b). Granulosa cell mRNA was assessed for each EP3 receptor (PTGER3) isoform and ACTB by real-time PCR (RT-PCR) using a Roche LightCycler (Roche). PCR was performed using the FastStart DNA Master SYBR Green I kit (Roche) following the manufacturer's instructions. Primers specific for each

cynomolgus macaque EP3 receptor (*PTGER3*) isoform and *ACTB* were designed using LightCycler Probe Design Software (Roche) based on the human or monkey sequences (Table 1). All primers span an intron to prevent undetected amplification of genomic DNA. A standard curve was generated for each primer set over at least five log dilutions using monkey cDNA of known copy number. For each sample, the copy numbers for each EP3 (*PTGER3*) isoform and *ACTB* mRNA were determined in independent assays. No amplification was observed when monkey cDNA was omitted. All data are expressed as the ratio of mRNA of interest to *ACTB* mRNA for each sample.

Granulosa cell subpopulations

Cumulus–oocyte complexes were mechanically removed from follicular aspirates. The remaining cells (mural granulosa cells) were enriched by Percoll gradient centrifugation as described earlier. Cumulus cells were removed from oocytes using stripper tips (Mid-Atlantic Diagnostics, Mt Laurel, NJ, USA). Each subpopulation of granulosa cells was frozen separately in liquid nitrogen and stored at -80°C for isolation of total RNA.

To obtain apex and non-apex granulosa cells of monkey ovarian follicles, whole stimulated monkey ovaries obtained before (0 h) and 36 h after hCG administration were bisected such that at least two follicles >4 mm in diameter were present on each piece. The ovarian pieces were covered with O.C.T. compound (Sakura, Tokyo, Japan), frozen in liquid propane, and stored at -80°C prior to sectioning at $10\text{ }\mu\text{m}$.

Laser capture microscopy (LCM) was used to obtain apex and non-apex granulosa cells as described previously (Harris *et al.* 2011). Briefly, monkey ovarian tissue sections were dehydrated as described by Arcturus HistoGene Frozen Section Staining Kit (Applied Biosystems) with the addition of a 2.5-min incubation with Nuclear Fast Red counterstain following distilled water incubations (Vector Labs). The granulosa cells adjacent to the thinnest area of the ovarian stroma were identified as apex mural granulosa cells while granulosa cells opposite the apex were identified as non-apex mural granulosa cells. These subpopulations of granulosa cells were captured onto CapSure Macro LCM caps (Molecular Devices) using an Arcturus AutoPix 100e LCM system (Mountain View, CA, USA).

Total RNA was isolated from mural and cumulus granulosa cells using the TRIzol method described earlier. For laser capture microdissection samples, the caps for apex and non-apex granulosa cells were dissolved in $200\text{ }\mu\text{l}$ TRIzol (Invitrogen) by inverting every 30 min for 1 h, and total RNA was obtained using the standard protocol for TRIzol. For all granulosa cell subpopulations, RNA amplification was conducted using the first amplification cycle in the Small Sample Labeling Protocol Version VII (small sample protocol, version II; Affymetrix, Santa Clara, CA, USA). This amplification was followed by RT of the T7 amplified RNA using M-MLV reverse transcriptase (Invitrogen). cDNA was then purified using Qiaquick PCR purification kit (Qiagen). mRNA levels for EP3 receptor (*PTGER3*) isoforms and *ACTB* in each subpopulation were assessed by RT-PCR as described earlier.

Statistical analysis

All data sets were assessed for heterogeneity of variance by Bartlett's test. Data were $\log_{10}$ transformed when Bartlett's test yielded $P<0.05$; log-transformed data were subjected to Bartlett's test to confirm that $P>0.05$. All data sets were assessed by *t*-test or ANOVA followed by Duncan's *post-hoc* test using StatPak Software, version 4.12 (Northwest Analytical, Portland, OR, USA). Significance was assumed at $P<0.05$. Data are presented as mean $\pm$ S.E.M.

Results

Identification of EP3 receptor isoforms expressed by monkey granulosa cells

After cDNA amplification and sequencing of the C-terminal region of each monkey EP3 isoform, the cDNA sequences were compared with human and available monkey sequences. Five EP3 isoforms were detected in monkey granulosa cells obtained throughout the ovulatory interval (Table 2). Monkey isoforms with high sequence homology to human EP3 isoforms are identified using previously reported nomenclature (Bilson *et al.* 2004). Monkey isoforms without high-sequence homology to previously reported isoforms are identified here as isoforms EP3-13 and EP3-14.

Table 1 Primer sequences for monkey EP3 receptor isoforms and *ACTB*.

Target	Sequence (5'–3')	Amplified cDNA (bp)	Published human sequence	Identity between monkey and human sequence (%)	Monkey amplified fragment accession no.
EP3-5	F: CCATTCAGCTCATGGGGA R: ACAGTAAGGTTTAGCATATTTG	388	NM_198715	98.6	KC154006
EP3-7	F: TGGTCTCCGCTACTGA R: ACAATTCCTCCTGGCAA	204	NM_198717	97	KC154007
EP3-9	F: CAACCTTGATGTGGAGT R: CCGCTTCGGTTAAGATT	382	NM_198719	95	KC154008
EP3-13	F: TTTGGGTTGCTTTACCT R: AGAGAGTCATGGAGCATC	201			KC154010
EP3-14	F: CCCACAACAACCTTGATG R: AGTGGCCCCAAATGTTA	267			KC154011
ACTB	F: ATCCGCAAAGACCTGT R: GTCCGCTAGAAGCAT	270	NM_001101	97.4	AY765990

Table 2 Carboxyl-terminal amino acid sequences for monkey EP3 receptor isoforms.

Isoform	C-terminal sequence
EP3-5	WVYLLLRKILLRKSCQVANAVSSCSNDGQKGQPISLSNEIIQTEA ^a
EP3-7	WVYLLLRKILLRKFCQEELWGN ^a
EP3-9	WVYXLLRKIXLRKFCQIRYHTNYYASSSTSLPCQCSSTLMWSDHFQRQ ^a
EP3-13	WVYLLLRKILLRKFCQANIPLYLYTPFSVSIYMSMDIWWAFTSWLL ^a
EP3-14	WVYLLLRKILLRKFCQGDPLEDCGLPSTELKILCSSELTAWRSKQRKTGTA ^a

^aStop codon.

Expression of functional EP3 receptor isoform proteins

CHO cells have been shown to express numerous G-proteins, so these cells are widely used to identify signal transduction pathways regulated by transfected GPCRs (An *et al.* 1994, Jin *et al.* 1997, Jakubík *et al.* 2006, Janíčková *et al.* 2013). CHO cells have been used as an *in vitro* cell model to study GPCRs and intracellular signaling that occurs in ovarian granulosa cells (Thiruppathi *et al.* 2001, Xing *et al.* 2006, Li *et al.* 2008). CHO cells were transiently transfected with a single HA-tagged EP3 isoform in the pcDNA3.1 vector in order to verify protein expression and determine the subcellular location of each EP3 receptor isoform (Fig. 1). In CHO cells expressing the EP3-5, EP3-9, and EP3-14 isoforms, fluorescence representing the EP3 receptor isoform was most prominent in the plasma membrane region. By contrast, fluorescence was present throughout CHO cells transfected with the EP3-7 and EP3-13 isoforms. These experiments were repeated using the pSI vector with the weaker promoter, and similar results were obtained (Fig. 1). Little or no staining was observed in CHO cells after mock transfection (not shown) or empty vector transfection (Fig. 1). Omission of primary antibody also resulted in little or no staining (Fig. 1, insets).

To identify G-proteins utilized by each monkey EP3 receptor isoform, CHO cells transfected with a single EP3 isoform in the pcDNA3.1 vector were treated with the EP3 receptor-selective agonist sulprostone, and the intracellular signals cAMP and calcium were measured.

Sulprostone stimulation of CHO cells expressing EP3-5 slightly but significantly reduced cAMP levels when compared with control (Fig. 2A). cAMP levels were not altered by the G α i inhibitor PTX alone, but sulprostone stimulation after pretreatment with PTX increased cAMP levels compared with sulprostone alone. Similarly, the general G-protein inhibitor GDP- β -S alone did not alter cAMP, but treatment with sulprostone after GDP- β -S increased cAMP above levels measured with sulprostone alone. Stimulation of EP3-5 by sulprostone did not alter intracellular calcium (Table 3). These findings are consistent with EP3-5 coupling to the G α i protein to regulate adenylyl cyclase and cAMP.

Sulprostone stimulation of CHO cells expressing EP3-9 increased cAMP levels when compared with control (Fig. 2B). GDP- β -S pretreatment reduced sulprostone-stimulated cAMP levels but did not alter control cAMP levels. PTX pretreatment did not alter control- or sulprostone-stimulated cAMP. These data suggest that EP3-9 couples with G α s to increase adenylyl cyclase

Figure 1 Subcellular localization of each EP3 receptor isoform. pcDNA3.1 (upper panels) and pSI (bottom panels) were used to determine the location of each monkey EP3 receptor isoform within CHO cells. Transient transfection with empty vector or vector containing a single EP3 receptor isoform with HA tag was performed. Cells were then fixed and stained with anti-HA tag antibody (green). Nuclei were stained with propidium iodide (red). No staining was observed when the primary antibody was omitted (insets). The results shown are representative of two independent experiments. All images are at the same magnification, with scale bar in upper left panel and inset (scale bars = 5 μ m).

Figure 2 EP3 receptor isoforms alter cAMP. CHO cells transiently transfected with *EP3-5* (A), *EP3-9* (B), or *EP3-14* (C) were treated with 0.01% DMSO (control) or sulprostone (Sulp) for 4 h. Cultured media were assessed for cAMP by EIA. Some cultures were pretreated with PTX or GDP- β -S (GDP) before addition of sulprostone. cAMP levels are presented relative to control, which is set equal to 1.0 for each independent experiment. Data are expressed as mean $\pm$ S.E.M., $n=4$ –10 independent experiments. Within each panel, data were assessed by ANOVA with one repeated measure, followed by Duncan's *post-hoc* test; groups with no common superscripts are different, $P<0.05$.

activity. Sulprostone treatment of CHO cells expressing EP3-9 also increased intracellular calcium (Fig. 3 and Table 3). This sulprostone-stimulated increase in intracellular calcium was blocked by pretreatment with

either PTX or GDP- β -S, supporting the concept that a PTX-sensitive G-protein mediates the ability of EP3-9 to regulate calcium.

Sulprostone stimulation of CHO cells expressing EP3-14 increased cAMP levels compared with control (Fig. 2C). PTX alone did not alter cAMP levels, but PTX pretreatment further increased sulprostone-stimulated cAMP levels. GDP- β -S alone did not alter cAMP levels, and pretreatment with GDP- β -S did not alter sulprostone-stimulated cAMP levels. Taken together, these observations suggest that more than one G-protein participates in the regulation of cAMP by EP3-14, which may include *G α i* as well as *G α s*. Sulprostone stimulation of CHO cells expressing EP3-14 did not alter intracellular calcium (Table 3). Sulprostone did not alter cAMP or intracellular calcium in CHO cells transfected with *EP3-7* and *EP3-13* (not shown).

EP3 isoform expression in monkey granulosa cells

To determine which EP3 receptor isoforms are regulated by gonadotropin during the ovulatory period, monkey granulosa cells obtained before (0 h) and 12, 24, and 36 h after hCG administration were assessed for each EP3 isoform mRNA by RT-PCR (Fig. 4). All isoforms showed low mRNA levels before (0 h) hCG. *EP3-5* and *EP3-7* isoforms increased 12 h after hCG treatment and maintained high levels through 36 h of hCG exposure. *EP3-9* mRNA reached peak levels 12 h after hCG administration but fell to moderate levels 24–36 h after hCG. *EP3-13* and *EP3-14* expression showed the same pattern as *EP3-5* and *EP3-7*, but these isoforms were expressed at levels tenfold lower than other EP3 isoforms.

To determine whether each functional EP3 receptor isoform has a unique temporal and spatial pattern of expression, granulosa cell subpopulations obtained before (0 h) and 36 h after hCG were assessed for expression of the functional EP3 isoforms: *EP3-5*, *EP3-9*, and *EP3-14*.

EP3-5 mRNA was significantly lower in apex granulosa cells compared with non-apex granulosa cells obtained 36 h after hCG (Fig. 5A). However, no differences were observed between apex and non-apex mural granulosa cell samples collected before hCG administration. Differences in *EP3-5* mRNA levels between mural and cumulus granulosa cells were not observed at 0 or 36 h after hCG (not shown).

EP3-9 mRNA was higher in cumulus than in mural granulosa cells at 36 h after hCG while no differences between cumulus and mural granulosa cells were measured before (0 h) hCG (Fig. 5B). *EP3-9* mRNA levels were not different between apex and non-apex granulosa cells at any time examined (not shown). Differences in *EP3-14* mRNA levels were not observed between mural and cumulus or between apex and non-apex granulosa cells (not shown).

Table 3 Regulation of intracellular calcium concentration via each monkey EP3 isoform.

Time after sulprostone treatment	EP3-5	EP3-7	EP3-9	EP3-13	EP3-14
0 s	1.00	1.00	1.00 ^a	1.00	1.00
40 s	1.01 ± 0.01	1.07 ± 0.08	1.21 ± 0.02 ^b	1.04 ± 0.03	1.06 ± 0.03
Ionophore	1.22 ± 0.02	1.41 ± 0.11	1.76 ± 0.16	2.00 ± 0.19	1.97 ± 0.20

All data are presented as mean ± S.E.M., $n = 3-7$ per group. ^{a,b}Differences between before (0 s) and 40 s after treatment ($P < 0.05$) by two-tailed, paired t -test.

Discussion

This study is the first to examine EP3 receptor isoform expression in the ovulatory follicle. Five EP3 receptor isoform mRNAs were detected in the granulosa cells of monkey ovulatory follicles. The sequences of three isoforms (EP3-5, EP3-7, and EP3-9) were over 95% identical to the sequences of the previously reported human isoforms (Bilson *et al.* 2004). Two other isoforms (EP3-13 and EP3-14) had previously unreported sequence insertions in the C-terminal domains or novel 3' ends when compared with previously reported human EP3 receptor isoforms. These unique sequences in the C-terminal domains may allow each EP3 isoform to localize to different areas within the cell and trigger different intracellular signaling pathways in response to ovulatory concentrations of PGE₂.

The C-terminal region of the EP3 receptor participates in receptor localization within the cell. Monkey EP3 receptor isoforms EP3-5, EP3-9, and EP3-14 localized predominantly in the plasma membrane of CHO cells, whereas EP3-7 and EP3-13 were located throughout CHO cells. Use of the pcDNA3.1 and pSI vectors resulted in the same distribution of each EP3 isoform within CHO cells, suggesting that cellular location was not simply the result of massive overexpression. These data are consistent with other studies of EP3 receptor isoforms and support the concept that the C-terminal region controls, at least in part, the subcellular localization of these receptors (Hasegawa *et al.* 2000, Bilson *et al.* 2004). Studies of human EP3 receptor isoforms show that they differ in subcellular localization depending on the sequence of their C-terminal regions (Bilson *et al.* 2004). For example, the human EP3.I isoform (or hEP3-4) was localized to the cell surface. While EP3 isoforms denoted as EP3.III (or hEP3-7), EP3.IV (or hEP3-6), and EP3.f (or hEP3-CRAe) were found close to the plasma membrane, EP3.V (or hEP3-CRAk) was localized to intracellular compartment(s). Upon stimulation with PGE₂, EP3.I rapidly translocated to cytoplasmic vesicles; EP3 isoforms II (or hEP3-5), V, VI, and f showed less complete movement to cytoplasmic vesicles than did EP3.I. By contrast, the EP3.III and EP3.IV isoforms did not translocate to cytoplasmic vesicles after PGE₂ treatment. A mouse EP3 isoform lacking the C-terminal region was observed exclusively in an intracellular compartment (Hasegawa *et al.* 2000), again supporting the concept that the

C-terminal region plays a pivotal role in directing the subcellular localization of EP3 receptor isoforms.

The amino acid composition in the C-terminal region of each EP3 receptor isoform is also crucial for interactions with G-proteins. To determine which G-proteins couple to each EP3 isoform, we assessed changes in cAMP and intracellular calcium, as these are the well-characterized second messengers close to G-proteins in signal transduction pathways. Stimulation of monkey EP3-5 with sulprostone decreased cAMP, likely via G α_i . Elevated cAMP with sulprostone and PTX is similar to previous reports of elevated cAMP with agonist and PTX in other cells, but the specific G proteins involved remain to be identified (Israel & Regan 2009, Talasila *et al.* 2009). Sulprostone stimulation of EP3-9 augmented cAMP concentrations, likely through G α_s . Notably, the EP3-9 isoform may also couple to a PTX-sensitive G-protein of the G α_i subfamily (Tomić *et al.* 2002) to increase intracellular calcium. Stimulation of EP3-14 by sulprostone elevated cAMP, indicating that the EP3-14 isoform also activates the G α_s protein. Pretreatment of CHO cells expressing EP3-14 with PTX increased

Figure 3 EP3-9 modulates intracellular calcium. CHO cells transiently transfected with the EP3-9 isoform were pretreated with no inhibitor (filled square), PTX (open square), or GDP-β-S (open circle); all cells were then incubated with the fluorescent calcium dye Fluo-4AM. Basal fluorescent emission was determined (0 s). Sulprostone was added, and fluorescent emission of the same cells was measured every 20 s. Finally, cells were exposed to the calcium ionophore A23187 (gray bar) as a positive control. Fluorescence is expressed relative to basal levels, which is set equal to 1.0 for each independent experiment. The mean of seven independent experiments is shown. Statistics are shown in Table 3.

Figure 4 EP3 receptor isoform mRNA levels in monkey granulosa cells during the ovulatory interval. EP3-5 (A), EP3-7 (B), EP3-9 (C), EP3-13 (D), and EP3-14 (E) mRNA were quantified within monkey granulosa cells obtained before (0 h), 12, 24, and 36 h after hCG administration. Each EP3 receptor isoform mRNA was expressed relative to ACTB. Data are expressed as mean $\pm$ S.E.M., $n=3-5$ monkeys per time point. With each panel, each EP3 isoform mRNA was assessed by ANOVA, followed by Duncan's *post-hoc* test. Groups with no common superscripts are different, $P<0.05$.

sulprostone-stimulated cAMP, but pretreatment with GDP- β -S did not alter cAMP level compared with sulprostone treatment alone, indicating a complex role for EP3-14 in regulating cAMP levels. Our data confirm and extend the findings of studies of EP3 receptor isoforms in that differences in amino acid composition of the C-terminal region result in coupling to different G-protein. In humans, EP3.II and EP3.IV isoforms increased cAMP via $G_{\alpha s}$ while EP3.I and EP3.II activated $G_{\alpha q}$ (Kotani *et al.* 1995). In mice, while EP3 α and EP3 β coupled to $G_{\alpha i}$ and resulted in reduction of cAMP, EP3 γ

coupled to both $G_{\alpha i}$ and $G_{\alpha s}$ (Irie *et al.* 1993, Sugimoto *et al.* 1993). Pretreatment of the mouse EP3 receptor isoform EP3 α with PTX blocked a PGE₂-induced increase in intracellular calcium, indicating that a PTX-sensitive G-protein was involved in the PGE₂-induced changes in intracellular calcium in this species as well (Irie *et al.* 1994). Previous studies by our laboratory demonstrated that EP3 stimulation of the total aspirated granulosa cell population obtained from monkey follicle aspirates just before ovulation increased cAMP and did not alter intracellular calcium (Markosyan *et al.* 2006). Total aspirated granulosa cells contain relatively few cumulus granulosa cells, and EP3-9 is expressed primarily in cumulus cells, perhaps explaining why sulprostone did not increase intracellular calcium in our previous study. In summary, monkey granulosa cells likely express primarily EP3-9 and/or EP3-14 proteins in response to the ovulatory dose of gonadotropin as these isoforms are capable of increasing cellular cAMP. However, these and other EP3 isoforms may be highly expressed in specific regions of the follicle and play locally important roles in the ovulatory response to PGE₂.

Figure 5 Expression of functional EP3 receptor isoforms in monkey granulosa cell subpopulations. EP3-5 (A) and EP3-9 (B) mRNA were quantified within apex, non-apex, mural, and cumulus granulosa cell subpopulations obtained before (0 h) and 36 h after hCG administration. Each EP3 isoform mRNA was expressed relative to ACTB. Data are expressed as mean $\pm$ S.E.M., $n=3-4$ monkeys per time point. Comparisons between subpopulations were performed using two-tailed, paired *t*-test (* $P<0.05$).

Differences in the temporal or spatial expression of EP3 isoforms within the follicle may be crucial for the proper ovulatory function of granulosa cells. Granulosa cells within the ovulatory follicle can be classified into subpopulations based on their location, histological characteristics, and functions in ovulatory events. For example, cumulus granulosa cells are specialized to support the oocyte and undergo expansion to facilitate oocyte release. *EP3* mRNA increased in bovine cumulus cells following the ovulatory gonadotropin surge, which plays a role in maintaining cumulus–oocyte complex quality (Calder *et al.* 2001). Our previous studies showed that *EP2* and *EP3* mRNA were expressed at higher levels in monkey cumulus cells than in mural cells at 36 h after hCG administration (Harris *et al.* 2011). *EP2* was previously shown to respond to agonist stimulation with increased cAMP (Markosyan *et al.* 2006). This study extends these findings to demonstrate that *EP3-9* expression is high in cumulus cells just before ovulation, and functional studies in CHO cells indicate that stimulation of *EP3-9* also increases cAMP. Therefore, cumulus expansion in monkey may be regulated by both *EP2* and *EP3-9* by responding to elevated follicular PGE_2 with increased cAMP. In the mouse, *Ep2* mRNA was highly expressed in cumulus cells just before ovulation (Segi *et al.* 2003, Takahashi *et al.* 2006), consistent with the concept that cumulus expansion is regulated by this G α s-coupled receptor. Mice lacking *Ep2* expression have a severe deficiency in cumulus expansion, partially via reduction of tumor necrosis factor α -induced protein 6 (*Tnfaip6*) selectively in cumulus cells of preovulatory follicles (Hizaki *et al.* 1999, Ochsner *et al.* 2003). *Tnfaip6* contains a hyaluronan-binding LINK domain and interacts with extracellular matrix components, including hyaluronan and inter- α trypsin inhibitor ($\text{I}\alpha\text{I}$), to stabilize the extracellular matrix during expansion of the cumulus–oocyte complex (Carrette *et al.* 2001, Mukhopadhyay *et al.* 2001). In rat and human granulosa cells, PGE_2 acts via the cAMP/PKA pathway to increase biosynthesis of epidermal growth factor (EGF)-like growth factors like amphiregulin and epiregulin (Ben-Ami *et al.* 2006, Nautiyal *et al.* 2010). These EGF-like growth factors participate in cumulus expansion by increasing expression of hyaluronan synthase 2 (*Has2*) and *Tnfaip6* (Park *et al.* 2004). These findings support the concept that *EP3-9* may cooperate with *EP2*, responding to PGE_2 with elevated cAMP within monkey cumulus cells, to regulate key proteins involved in cumulus expansion.

PGE_2 -dependent events associated with follicle rupture are likely mediated in part via *EP3-5*. Both *EP1* (Harris *et al.* 2011) and *EP3-5* (present study) are expressed at lower levels at the follicle apex than in non-apex granulosa cells just before ovulation. Our laboratory previously showed that *EP1* and *EP3* increased SERPINE1, which was present at higher levels in non-apex mural granulosa cells than in apex cells (Markosyan & Duffy 2009, Harris *et al.* 2011). SERPINE1 is an endogenous

inhibitor of PLAT activity, a proteolytic pathway implicated in degradation of the follicle apex at ovulation (Strickland & Beers 1976, Liu *et al.* 2004). High levels of SERPINE1 mediated by elevated intracellular calcium via *EP1* may protect non-apex mural granulosa cells from the proteolytic action of PLAT, the primary PLAT in the monkey follicle at ovulation (Markosyan & Duffy 2009). We have also previously shown that *EP2* and *EP3* increase expression of PLAT in monkey granulosa cells (Markosyan & Duffy 2009). The primary intracellular signal generated by PGE_2 stimulation of these receptors in monkey granulosa cells is cAMP (Markosyan *et al.* 2006). However, this study shows that *EP3-5* is expressed at low levels in granulosa cells at the follicle apex. As functional studies in CHO cells indicate that *EP3-5* decreases cAMP levels, PGE_2 stimulation of this *EP3* receptor isoform may result in reduced PLAT expression in non-apex granulosa cells, with relatively higher PLAT levels in granulosa cells at the follicle apex. Therefore, low expression of *EP1* and *EP3-5* at the follicle apex may result in high PLAT activity in apex mural granulosa cells and participate in defining the site of follicle rupture.

Differential expression of each *EP* receptor, and now each *EP3* receptor isoform, in subpopulations of granulosa cells may contribute to the different roles that these subpopulations play in the process of ovulation. Functional studies in CHO cells indicate that monkey *EP3* isoforms interact with different G-proteins to activate distinct combinations of intracellular signaling pathways. *EP3* isoforms also have different temporal and spatial distributions throughout the follicle. These differences suggest that granulosa cells can generate a unique response to PGE_2 dependent on gonadotropin exposure, location within the follicle, and a unique pattern of *EP* receptor expression. Regional responses to PGE_2 include patterns of gene expression that define the different ovulatory functions of granulosa cells, such as maintenance of oocyte health, expansion of the cumulus, or proteolysis at the follicle apex. By understanding the role of each *EP* receptor (including each *EP3* isoform) in regulating ovulatory functions of granulosa cells, receptor-selective agonists and/or antagonists may be utilized to promote fertility, prevent ovulation, or create new contraceptives.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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