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The G-protein-coupled receptors GPR3 and GPR12 are involved in cAMP signaling and maintenance of meiotic arrest in rodent oocytes

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Abstract

In mammalian and amphibian oocytes, the meiotic arrest at the G2/M transition is dependent on cAMP regulation. Because genetic inactivation of a phosphodiesterase expressed in oocytes prevents reentry into the cell cycle, suggesting autonomous cAMP synthesis, we investigated the presence and properties of the G-protein-coupled receptors (GPCRs) in rodent oocytes. The pattern of expression was defined using three independent strategies, including microarray analysis of GV oocyte mRNAs, EST database scanning, and RT-PCR amplification with degenerated primers against transmembrane regions conserved in the GPCR superfamily. Clustering of the GPCR mRNAs from rat and mouse oocytes indicated the expression of the closely related *Gpr3*, *Gpr12*, and *Edg3*, which recognize sphingosine and its metabolites as ligands. Expression of these mRNAs was confirmed by RT-PCR with specific primers as well as by in situ hybridization. That these receptors are involved in the control of cAMP levels in oocytes was indicated by the finding that expression of the mRNA for *Gpr3* and *Gpr12* is downregulated in *Pde3a*-deficient oocytes, which have a chronic elevation of cAMP levels. Expression of GPR3 or GPR12 in *Xenopus laevis* oocytes prevented progesterone-induced meiotic maturation, whereas expression of FSHR had no effect. A block in spontaneous oocyte maturation was also induced when *Gpr3* or *Gpr12* mRNA was injected into mouse oocytes. Downregulation of GPR3 and GPR12 caused meiotic resumption in mouse and rat oocytes, respectively. However, ablation of the *Gpr12* gene in the mouse did not cause a leaky meiotic arrest, suggesting compensation by *Gpr3*. Incubation of mouse oocytes with the GPR3/12 ligands SPC and S1P delayed spontaneous oocyte maturation. We propose that the cAMP levels required for maintaining meiotic arrest in mouse and rat oocytes are dependent on the expression of *Gpr3* and/or *Gpr12*.

Keywords: Oocyte; Meiosis; cAMP; G-protein-coupled receptors; Mouse; Rat

Introduction

In the fully developed antral follicles of the mammalian ovary, oocytes are arrested in meiotic prophase in spite of being fully competent to resume meiosis (Eppig, 1993; Tsafriri and Dekel, 1994). Removal of the oocytes from their follicular environment causes resumption of meiosis and maturation (Pincus and Enzmann, 1935) to yield an egg fully competent for fertilization and embryo development (Eppig, 1993; Tsafriri and Dekel, 1994). The LH surge is the signal that triggers oocyte reentry into the cell cycle prior to ovulation. Because LH receptors are present only on the granulosa cells of the follicle, intermediate steps are required to relay the gonadotropin signal from somatic cells to the oocyte (Park et al., 2004).

It is generally accepted that cAMP signaling plays an important role in maintaining this meiotic arrest (reviewed in Conti et al., 2002). This hypothesis was initially based on pharmacological manipulation of cAMP levels in oocytes during in vitro spontaneous maturation (Cho et al., 1974; Dekel and Beers, 1978; Schultz et al., 1983; Vivarelli et al., 1983) and in vivo maturation induced by the LH surge (Wiersma et al., 1998). More recently, it has been shown that injection of inhibitory Gs antibodies into mouse oocytes still enclosed in the follicle causes germinal vesicle breakdown (GVBD) (Mehlmann et al., 2002). This seminal observation demonstrates that oocytes are autonomous in terms of cAMP production and implies that an active Gs protein and adenylyl cyclase are required to maintain meiotic arrest. Indeed, genetic

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evidence has shown that ablation of the *Adcy3* adenylyl cyclase gene in the mouse produces a leaky meiotic arrest (Horner et al., 2003). In the same vein, genetic ablation of the major PDE expressed in mouse oocytes causes complete female sterility due to ovulation of immature GV-arrested oocytes that cannot be fertilized by spermatozoa (Masciarelli et al., 2004).

Pde3a-deficient oocytes do not undergo meiotic maturation even after prolonged incubation in vitro or after ovulation and transfer to the ampulla, suggesting that the meiotic block is maintained autonomously by the oocyte outside the follicle (Masciarelli et al., 2004). This block is most likely due to constitutive cAMP signaling because increased cAMP in these oocytes was associated with undetectable cAMP-PDE activity. That this block is dependent on the presence of an active adenylyl cyclase and Gs protein is suggested by the observation that inhibition of Gs by injection of a Gsα inhibitory antibody in the *Pde3a*-deficient oocytes causes GVBD and a prompt reentry into the cell cycle (Mehlmann and Jaffe, personal communication). Similarly, inhibition of steps downstream of cAMP, such as PKA catalytic activity, restores meiotic maturation in these oocytes (Masciarelli et al., 2004).

Taken together, the above findings strongly suggest that meiotic arrest is dependent on continuous cAMP signaling through active Gs/adenylyl cyclase endogenous to the oocyte. An extension of this hypothesis is that one or more receptors involved in the regulation of cAMP are expressed in oocytes. Since these receptors should signal through a Gs protein, they likely belong to the family of GPCRs with the characteristic seven transmembrane domain motifs. Given the continuous cAMP accumulation and meiotic arrest of the *Pde3a*-null oocytes even after removal from the follicle and the observation that a Gsα inhibitory antibody promotes maturation in denuded oocytes, we speculated that these GPCRs have significant activity toward Gs even in the absence of a putative ligand produced by the somatic cells of the follicle.

To test this possibility, we have used several independent approaches to define the pattern of GPCR expression in rodent oocytes arrested in meiotic prophase. Here, we show that a subfamily of GPCRs that bind the ubiquitous lysophospholipid mediators is expressed in GV stage mouse and rat oocytes. These receptors show constitutive activity toward Gs when expressed in a heterologous system, and their overexpression blocks oocyte maturation in *Xenopus* and mouse oocytes. Major differences in the expression and function of GPR3 and GPR12 were found in mouse and rat, suggesting that their involvement in meiotic arrest is species-specific.

Materials and methods

Animal treatments, collection of oocytes, cumulus cells, and granulosa cells

All animal procedures were in accordance with accepted standards of humane animal care and were approved by the Institutional Animal Care and Use Committee at Stanford University.

Immature (23-day-old) C57Bl/6 mice or Sprague–Dawley rats were primed with 5 or 10 IU PMSG, respectively. After 45 h, ovaries were excised in L-15 Liebovitz's medium (L-15) (Invitrogen, Carlsbad, CA) with 5% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 U/ml streptomycin. FBS was substituted with 0.7% polyvinylpyrrolidone (PVP) (Sigma, St. Louis, MO) for ligand studies or with 0.3% fatty acid-free BSA (FAF-BSA) (Sigma) for injection studies. Antral follicles were punctured with a needle, and cumulus–oocyte complexes (COCs) were aspirated and stripped of cumulus cells by repeated pipetting with a small bore pipette. Denuded oocytes were washed free of all cells by transferring to successive dishes of medium. For cumulus cell collection, COCs were separated from mural granulosa cells and then stripped. Granulosa cells were collected from the punctured ovaries after removing the ovaries and COCs.

Gpr12-deficient mice were generated by Deltagen (Palo Alto, CA; construct T341). A deletion of approximately 500 bp was introduced by insertion of the IRES-lacZ reporter and neomycin resistance cassette. The *Gpr12*-null allele of the mice used in our studies was maintained on a C57Bl/6J background. Mice were genotyped by extracting tail DNA using two PCR reactions designed to detect wild-type and targeted alleles.

Screening for GPCR mRNA expression in rodent oocytes

Oocytes were collected from PMSG-treated immature C57Bl/6 mice and Sprague–Dawley rats as described above. Denuded oocytes (2000) were used for RNA extraction within 30 min after follicle puncture following the protocol for TRIzol reagent (Invitrogen). RNA was quantified by optical density at 260 nm, and RT reactions were performed with approximately 100 ng of RNA using random hexamers. The cDNA thus obtained was used for RT-PCR with the following primers designed on the basis of the conservation of sequences in transmembrane domains 3 (TM3) and 6 (TM6) of GPCRs (TM3 = 5′-CT(G/C) CT(G/C) G(C/T)(C/G) AT(C/T) GCI (G/C)T(G/C) GA(C/T) (C/A)GI TA-3′; TM6 = 5′-(G/A)(A/T)A IGG (C/G)AG CCA (G/A)CA (G/C)A(T/C) IG(C/T) (G/A)AA-3′. The PCR conditions were as follows: 30 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 3 min, then elongation at 72°C for 7 min. The amplified fragments corresponding to approximately 450 bp were purified from a 1.5% agarose gel and subcloned into pCR2.1-TOPO vector (Invitrogen). Random colonies were picked, plasmid purified, and used for sequencing. The nucleotide sequences were performed by Biotech Core (Mountain View, CA) with an ABI automated DNA sequencer. Sequences were analyzed for identity using the CLUSTAL algorithm, and the identities were determined by BLAST analysis.

Murine Genome U74Av2 GeneChips microarray data generated using GV or MII oocyte RNA were obtained from Wang et al. (2004). Records were scanned for G-protein-coupled receptor entries. The P call% was ignored for genes coding for GPCR whose expression levels were calculated above the 1000 value (Wang et al., 2004). Entries that were below 1000 and called absent by the Affimetrix program were not included.

The following data sets of expressed sequence tags (dbEST) derived from mouse oocyte and egg cDNA libraries were searched: Mouse GV Oocyte Libraries NIH_MGC_256 and NIH_MGC_257, dbEST Library 10029, NIA Mouse Unfertilized Egg cDNA Library (Long), dbEST Library 14142, NIA Mouse Unfertilized Egg cDNA Library (Long 1), and dbEST Lib.1389 Mouse Unfertilized Egg cDNA Library. Using this strategy, ESTs corresponding to the following GPCRs were retrieved: *Gpr1*, *Gpr3*, *Gpr35*, *Gpr56*, *Gpr108*, *Gpr116*, *Gpr125*, *Gpr133*, *Oprm1*, and *Lgr6*. The EST accession numbers which overlap with the microarray data are reported in Table 1.

RT-PCR with GPCR-specific primers

To confirm the presence and compare the expression of each gene in the different cell types of the ovary, the Cell-to-cDNA kit (Ambion, Austin, TX) was used to perform RT-PCR on 60 denuded oocytes from mouse and rat and on cumulus and mural granulosa cells from 6 mouse ovaries. The cDNA from the three cell types was prepared from four different cell collections and normalized to *β-actin*. In several experiments, 1000–2000 oocytes from wild-type and *Pde3a*-deficient mice were also collected, and total RNA was extracted according to the protocol for TRIzol reagent. RT reactions were performed with 0.3–1 μg of RNA using random hexamers. The quantity of RNA was measured by optical density at 260 nm, and the amount of cDNA used in the PCR was normalized to *β-actin*. To control for genomic DNA contamination, PCRs were performed with gene-specific primers on RNA without reverse transcriptase treatment. The cDNA prepared from rat and

Table 1
GPCR gene expression in mouse oocytes

Gene name	Description	Accession number	Alternative names	G-protein coupling	Ligands	Microarray mRNA ± SEM	ESTs	RT-PCR
Adora2a	Adenosine A2a receptor	NM009630	Adora2a A2aR	G_s	Adenosine	901 ± 100	CO807420.1	Present
Mc1r	Melanocortin 1 receptor	X65635	Mc1r, e, Tob, Mshra tobacco darkening	G_s	MSH	709 ± 85		
Mc5r	Melanocortin 5 receptor	U08354	Mc5r	G_s	MSH	1162 ± 132		
Gpr3	G-protein-coupled receptor 3	D21062	GPCR 21,ACCA	G_s,$G_{q/11}$,G_i	S1P	4594 ± 432	BB709334	Present
Gpcr12	G-protein-coupled receptor 12	D21061	GPCR01	G_s,$G_{q/11}$,G_i	SPC,S1P, DHS1P	8408 ± 376		Present
Edg3	EDG-3	AF108021	LPb3, S1P3	$G_{q/11}$, $G_{i/o}$	S1P,DHS1P	1983 ± 268		Present
Gpr50	G-protein-coupled receptor 50	AF065145	H9, melatonin-related receptor		?	4734 ± 304		
Adrb2	Adrenergic receptor, beta 2	X15643	Adrb2,Badm, Gpcr7, Adrb-2, beta 2-AR	Gs	Catecholamine	1329 ± 156		
Htr5a	5-Hydroxytryptamine (serotonin) receptor 5A	Z18278	Htr5	$G_{i/o}$	Serotonin	873 ± 81		
Hrh1	Histamine receptor H1	D50095	Bphs, Bordetella pertussis-induced histamine sensitization	G_s	Histamine	831 ± 146		
Gpr27	G-protein-coupled receptor 27	AF027955	Sreb1		?	2388 ± 226		
Ptger2	Prostaglandin E receptor EP2 subtype	D13458	Prostaglandin E receptor2 (EP4 subtype), Pter4		Prostaglandin E	433 ± 125		
Npy2r	Neuropeptide Y receptor Y2	D86238	NPY-Y2 receptor	$G_{i/o}$	Neuropeptide Y	247 ± 36		
Cckar	Cholecystokinin A receptor	D85605		G_s,$G_{q/11}$	CCK-A	369 ± 41		
Cckbr	Cholecystokinin B receptor	AF019371	Cck2/gastrin receptor, Cck-B/gastrin receptor	$G_{q/11}$	CCK-B	2453 ± 114		
Sstr4	Somatostatin receptor 4	U26176	Sst4, Smstr4	$G_{i/o}$	Somatostatin	3070 ± 200		
Oprd1	Opioid receptor, delta 1	L11064		$G_{i/o}$	Opioid	695 ± 88		
Oprs1	Opioid receptor, sigma 1	AF004927	mSigmaR1		Opioid	1697 ± 180		
Cxcr3	Chemokine (C-X-C) receptor 3	AF045146	CXCR3, Cd183, Cmkar3		Chemokine	4053 ± 172		
Bdkrb2	Bradykinin receptor, beta	L26047	*kinin B2, B2, B2R,*		Bradykinin	2781 ± 303		
Cmkor1	Chemokine orphan receptor 1	AF000236	Rdc1		Chemokine	356 ± 43		
Admr	Adrenomedullin receptor	D17292	NOW, AM-R, MB10, G10-D, Gpcr17,Gpcr22		Adrenomedullin	1100 ± 125		
Gpr44	G-protein-coupled receptor 44	AF054507	Crth2, Gpr45			2328 ± 259		
Olfr157	Olfactory receptor 37c	AJ133426	Olfactory rec. 157 OR37C, mOR37c			1219 ± 133		
Olfr158	Olfactory receptor 37d	AJ133427	Olfactory rec. 158, mOr37d, Olfr37d			2198 ± 183		
Olfr71	Olfactory receptor 71 (17)	AJ133429	Olfactory rec. 71, mOr17, Mor262-4			7561 ± 899		
Gpr19	G-protein-coupled receptor 19	U46923				3380 ± 374		
Gcgr	Glucagon receptor	L38613	Gr	G_s	Glucagon	2640 ± 79		
Ghrhr	Growth hormone releasing hormone receptor	L07379	Ghrhr, lit, Ghrfr	G_s	GHRH	791 ± 99		
Gpr56	G-protein-coupled receptor 56	BC021648	Serpentine receptor			6966 ± 826	CO797582.1	Present
Grm8	G-protein-coupled receptor, family C, group 1, member H	U17252	Glutamate receptor, metabotropic 8, Gprc1h	$G_{i/o}$	Glutamate	582 ± 47	CO809126.1, CO804286.1	

The levels of mRNA expression for each GPCR were determined by analyzing a microarray data set of RNAs from GV oocytes hybridized to Murine Genome U74Av2 GeneChips (Wang et al., 2004). The microarray data used to generate this table are present in the public database ArrayExpress (http://www.ebi.ac.uk/arrayexpress). When the expression of a GPCR was detected both through EST database scanning and microarray analysis, the matching EST accession number is indicated in the corresponding column. The last column indicates the GPCRs that were detected by RT-PCR of mouse and/or rat GV oocyte mRNA.

mouse brain was used as positive controls in the PCRs comparing expression of GPCRs in the two species. For amplification of GPCRs, gene-specific primers 5′-GACATTGTCTTGTGCAGCTCAGGA-3′ and 5′-GCGTAAATGACAGGGTTGATGATGGA-3′ were used to confirm the presence of *Gpr12*; primers 5′-GTGGTGCTGTGCATCTCAGGCA-3′ and 5′-GGTTGATCATGGAGTTGTAGGT-3′ were used for *Gpr3*; primers 5′-CTGGCCATTGCCATTGAGCGACAC-3′ and 5′-ACCAACAGGCAATGAACACACTCA-3′ were used for *Edg*-3; and primers 5′-GAGGATCTGCATATCCACTCCCAG-3′ and 5′-GAGTGGCTTCTACCTCTGTGGATG-3′ were used for *Gpr56*. The PCR conditions were a hot start at 94°C for 3 min and 35 cycles of 94°C for 1 min, 62°C for 1 min, and 72°C for 1 min, and 72°C for 6 min. To confirm the identity of the receptors, bands of 763, 725, 317, and 455 bp for *Gpr12*, *Gpr3*, *Edg3*, and *Gpr56*, respectively, were purified from agarose gels, cloned into pCRII-TOPO vectors (Invitrogen), and sequenced. For semiquantitative analysis, the number of amplification cycles was chosen in the exponential phase of amplification for each primer pair. These experiments evaluating the expression of *Gpr3* and *Gpr12* were repeated with additional pairs of primers, 5′-TCTATGGCCTGGTTCTCAGCTG-3′ and 5′-GCATTGTACAGGAAAGGTAGCG-3′, and 5′-CACCAAGCTGGTCACCATCG-3′ and 5′-GCGTGCCTCATCACAATCTTACA-3′, respectively. PCR products visualized on ethidium bromide–agarose gels were analyzed using NIH Image (Scion, Frederick, MD) to compare the densities of the bands.

Maturation studies

Sphingosylphosphorylcholine (SPC) (Biomol, Plymouth Meeting, PA) was dissolved in water, and sphingosine-1-phosphate (S1P) (Biomol) and sphingomyelin (SM) (Biomol) were prepared as liposomes, according to the manufacturer's instructions. Denuded oocytes were collected in L-15 medium with 0.7% PVP with or without 1 or 10 μM SPC or S1P or 10 μM SM and placed in 25 μl drops of the same medium under mineral oil. From the time of puncture of the follicles, the oocytes were scored for meiotic maturation every 15 min for 2 h.

Progression of meiotic maturation of denuded mouse oocytes was scored by monitoring the breakdown of germinal vesicles (GVBD) with an inverted microscope (Olympus, Melville, New York) fitted with a Hoffman contrast lens. Oocytes showing clear nuclear membranes (germinal vesicle, GV) and nucleoli were classified as GV stage; those without visible nuclear structure, i.e., exhibiting germinal vesicle breakdown (GVBD), were classified as GVBD stage; and metaphase II-arrested oocytes, with a polar body (PB), were classified as MII. Time 0 was the time of follicle puncture.

In situ hybridization

Ovaries of 25-day-old PMSG-treated mice and rats were fixed in 4% paraformaldehyde for 6 h followed by incubation in 0.5 M sucrose in PBS overnight at 4°C as previously described (Park et al., 2003). The ovaries were embedded in OCT (Tissue-Tek, Redding, CA), cut into 10 μm sections, and mounted on Superfrost slides (Fisher, Pittsburgh, PA). Slides were prepared for hybridization as previously described (1). The *Gpr3* and *Gpr12* cDNA (725 and 763 bp, respectively) were subcloned into pCRII-TOPO vector (Invitrogen), linearized, and transcribed to synthesize [35S]-labeled RNA probes. Hybridization mixtures with antisense and sense RNA probes were added to the slides and incubated overnight at 65°C. Post-hybridization washes consisted of RNaseA treatment and decreasing concentrations of SSC washes. Hybridized slides were then dehydrated and dried. Slides were dipped into NTB2 Emulsion (Eastman Kodak, Rochester, NY), exposed for 1–8 days, developed photographically, and then counterstained with Harris Hematoxylin and EosinY (0.125% in ethanol). Following counterstaining, tissues were cleared with xylene, mounted with Permount (Fisher, Fair Lawn, NJ), visualized, and photographed with AxioCam (Zeiss, Oberkochen, Germany).

Cloning of mouse Gpr3 and Gpr12 cDNA into mammalian expression vectors and transient transfection in COS7 cells

Full-length cDNA of murine *Gpr3* was amplified by PCR from mouse brain cDNA and cloned into *pcDNA3-V-5* or *pGEMHE* vectors. Primers used were the gene-specific primers 5′-ACCATGATGTGGGGAGCAGGAAGCT-3′ and 5′-GTAGACATCACTAGGGGACCGGGA-3′. Clones for *pGEMHE-Gpr12* with and without signal peptide (SP) were provided by Dr. Chica Schaller (Ignatov et al., 2003) and were subcloned into pcDNA3-V-5 for mammalian expression. All constructs were sequenced to ensure correct insertion and sequence.

COS7 cells were cultured at 37°C in a 5% CO_2 atmosphere in DMEM (Dulbecco's modified Eagle's medium) (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 U/ml streptomycin. For recombinant expression of GPCR constructs in COS7, cell cultures were grown on 6-well plates to 30% confluency and were then transfected with the following recombinant plasmids using the Effectene transfection system (Invitrogen) according to the manufacturer's protocol with 0.5 μg of DNA: *pCDNA3*, *pCDNA3-SP-Gpr12*, *pcDNA3-SP-Gpr3*, *pcDNA3-hfshr*, or *pcDNA3-Adrb2* (β-adrenergic receptor). Two days after transfection, the medium was changed to DMEM without serum. After a further 24 h, cells were incubated in medium with 0.5 mM IBMX with varying concentrations of SPC or S1P (0.1–10 μM) for cells transfected with *Gpr12* or *Gpr3*, or with hFSH (100 ng/ml) added to the *Fshr* transfected cells. Cells were harvested for cAMP quantification after 30 min.

Measurement of cAMP content in COS7 cells

Transfected cells were scraped in 1 ml of cold 0.1% TCA in 95% EtOH followed by centrifugation at 3000 rpm for 30 min at 4°C. The supernatant was evaporated and reconstituted in 1 ml PBS. After acetylation, cAMP content was measured by radioimmunoassay (Harper and Brooker, 1975). The pellet was dissolved in 0.5 ml 1% NaOH, and the protein content was determined by a modified Lowry colorimetric assay (Bensadoun and Weinstein, 1976).

Injection of RNA into mouse oocytes

Capped mRNAs were prepared from *pEgfp* and full-length coding regions of *mGpr3* and *mGpr12* cloned into pcDNA3 with the mMessage mMachine kit (Ambion), purified with RNeasy kit (Qiagen, Valencia, CA), and diluted to 0.5 μg/μl. Using an IM-300 Microinjector (Narishige International, East Meadow, NY) and sterile pipettes, 10–15 pl of mRNA was microinjected into the cytoplasm of denuded GV oocytes in M2 medium (Specialty Media, Phillipsburg, NJ) with 0.3% FAF-BSA and 10 μM cilostamide (Sigma). Following microinjection, oocytes were transferred to α-MEM medium (Invitrogen) with 10 μM cilostamide and 0.3% FAF-BSA and incubated for 4 h at 37°C in 5% CO_2. After incubation, morphologically normal oocytes were washed extensively in L-15 medium with 0.3% FAF-BSA without cilostamide and cultured for 24 h at 37°C in humidified air. The meiotic progression was scored every 15 min for the first 4 h using an Olympus inverted microscope fitted with a Hoffman contrast lens at 200×.

Injection of morpholino oligonucleotides in mouse and rat oocytes

For targeted knockdown of the GPR3 and GPR12 proteins, antisense morpholino oligonucleotides (MO) (*Gpr3MO* and *Gpr12MO*) (mouse *Gpr3*: 5′-AGCTTCCTGCTCCCCACATCATG-3′, rat *Gpr3*: 5′-GTCTTCGTTCATTTTAACCCCTGTC-3′ and mouse/rat *Gpr12*: 5′-GTCTTCGTTCATTTTAACCCCTGTC-3′), corresponding to 25 nucleotides surrounding the ATG initiation codon of mouse *Gpr3* and *Gpr12* (NM_008154 and NM_008151, respectively) and rat *Gpr3* and *Gpr12* (NM_153727 and NM_030831, respectively), were purchased from Gene Tools (Philomath, OR). The standard control morpholino oligonucleotide with the sequence 5′-CCTCTTACCTCAGTTACAATTTATA-3′ (Gene Tools) was used for control injections. All oligonucleotides were dissolved in nuclease-free water at a concentration of 1 mM and stored at −80°C in 2 μl aliquots. 10–15 pl of the indicated MO was injected into the cytoplasm of the oocytes in M2 medium supplemented with 0.3% FAF-BSA and 4.5 mM hypoxanthine. Immediately after injection, oocytes were transferred to α-MEM medium supplemented with 0.3% FAF-BSA and 4.5 mM hypoxanthine, incubated at 37°C in 5% CO_2, and meiotic progression was scored up to 48 h.

Expression of GPR3 and GP12 by injection of RNA into Xenopus oocytes

Ovary fragments were surgically removed from PMSG-primed *X. laevis*, and defolliculated oocytes were isolated after treatment with collagenase (2.5 mg/ml) in MBS buffer [10 mM HEPES (pH 7.4), 88 mM NaCl, 1 mM KCl, 0.82 mM $MgSO_4$, 2.4 mM Na_2 HCO_3] for 1–1.5 h. Dumont Stage VI oocytes were selected for all experiments. Oocyte selection and experiments were carried out in OR2 solution [5 mM HEPES (pH 7.8), 82.5 mM NaCl, 2.5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM Na_2HPO_4]. *Gpr12* and *Gpr3* cRNA were in vitro transcribed from the pGEMHE+SP vector using T7-RNA-polymerase and a *Nhe*I-linearized plasmid. The mRNAs or H_2O (vehicle) were injected using a micromanipulator (Narishige USA, Long Island, NY) into defolliculated *Xenopus* oocytes. Sixteen hours after injecting increasing concentrations of *Gpr12* or *Gpr3* mRNA (ranging from 0.2 to 5 ng/oocyte in 50 nl), the oocytes were stimulated with 500 nM progesterone. Resumption of meiosis was scored every hour, up to 10 h, by the appearance of a white spot on the animal pole of the oocyte.

Statistical analyses

Values were compared by Student's t test, and $P < 0.05$ was considered statistically significant.

Results

Screening for mRNAs coding for GPCRs expressed in mouse and rat oocytes

Three independent strategies were used to identify GPCR mRNAs expressed in rodent GV oocytes. Degenerate primers corresponding to the most conserved regions of transmembrane domains 3 and 6 in the GPCR families (Fredriksson et al., 2003) were designed and used for RT-PCR with rat or mouse oocyte RNA. In addition, microarray data from mouse GV and MII oocytes were searched for GPCR RNA expression (Wang et al., 2004). Finally, available EST databases generated by sequencing of mouse oocyte libraries, including data from a library that we have recently generated (NIH_MGC_256, NIH_MGC_257), were used for GPCR data mining. RT-PCR with degenerate primers on rat GV oocyte mRNA yielded sequences corresponding to *Adora2a* (adenosine A2a receptor) and to *Gpr12*, a receptor that binds lysophospholipids (Ignatov et al., 2003). The expression of these mRNAs in the rat oocyte was consistent with the data retrieved by microarray and EST data mining of mouse oocyte libraries. A summary of the GPCR mRNAs retrieved by these three methods is reported in Table 1.

From this survey, it is evident that GPCRs corresponding to different families, including family 1 and family 2, as well as the olfactory receptor family, are likely expressed in the quiescent oocyte (Table 1). Some of the receptors such as *Gpr56*, *Gpr44*, and *Gpr19* are orphan receptors with unknown ligands (Liu et al., 1999). The expression of the adenosine receptor A2a/b mRNA is consistent with previous reports of the effects of adenosine on oocytes (Salustri et al., 1988; Tornell et al., 1990; Eppig et al., 1985). In addition, expression of mRNA for *Gpr56* in oocytes has been previously determined (Stanton and Green, 2001). This receptor belongs to the family of secretin receptors, even though a ligand has not been identified and a possible function in oocytes has not been explored (Liu et al., 1999).

Analysis of GPCR families expressed by homology clustering showed that *Gpr3*, *Gpr12*, and *Edg3* and alpha MSH receptors belong to the same subfamily (Fig. 1). Whereas melanocortin receptors 1 and 5 use peptides as ligands, GPR3, GPR12, and EDG3 are receptors for the lipids S1P and SPC (Kostenis, 2004). In view of their properties and their binding to ligands produced locally and ubiquitously, we focused on the expression and function of GPR3, GPR12, and EDG3 in oocytes.

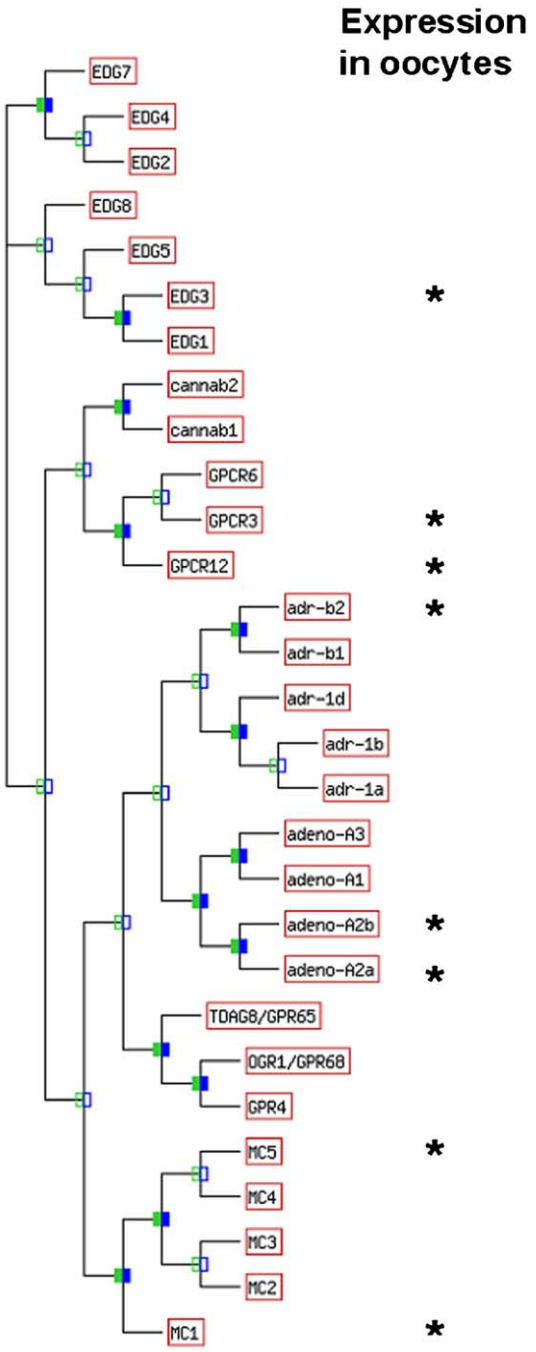

Fig. 1. Phylogenetic analysis of mouse S1P family of receptors and closely related receptors expressed in mouse oocytes. The phylogenetic tree was generated by CLUSTAL/W and BLOCK Maker using the mouse sequences of the eight *Edg* receptors. Branch length indicates the relatedness of two sequences. Asterisks indicate the members of the different families expressed in oocytes.

Expression of Gpr3, Gpr12, and Edg3 in somatic and germ cell compartments of rodent ovaries

To further investigate the expression of these receptors in different cells of the ovarian follicle, RT-PCR was performed with specific primers based on the mRNA sequences of *Gpr3*, *Gpr12*, and *Edg3*. *Gpr56* was used for comparison. RT-PCRs were performed on RNA from mouse oocytes, cumulus, and mural granulosa cells after RNA extraction (data not shown) or using procedures that do not require the isolation of RNA (Figs. 2A, B). Both methods yielded similar qualitative results. Unlike *Gpr56*, which is expressed in all three cellular compartments, *Gpr3*, *Gpr12*, and *Edg3*, showed specificity in their pattern of expression. Whereas *Gpr12* is expressed exclusively in oocytes, with undetectable expression in somatic cells, *Gpr3* and *Edg3* mRNAs are expressed mainly in oocytes and to a lesser extent in cumulus cells. Comparison by density measurements of the PCR products on ethidium bromide-stained agarose gels indicated that the expression of *Gpr3* and *Edg3* in cumulus cells is approximately 10% of that found in oocytes. In addition, semiquantitative comparison of *Gpr3* and *Gpr12* expression in mouse oocytes indicated that *Gpr3* mRNA is about fourfold more abundant than *Gpr12*. On the contrary, only *Gpr12* expression was observed when rat oocytes were used (Fig. 2C). This cellular pattern of expression within the follicle was confirmed by in situ hybridization with *Gpr3* (Figs. 3A, B) and *Gpr12* (Figs. 3C, D) antisense probes in mouse and rat ovaries, respectively. Major signal was observed in the oocytes from follicles at different stages of development, with *Gpr3* being readily detectable in mouse oocytes and *Gpr12* in rat oocytes. *Gpr3* expression could not be detected in rat ovaries (data not shown), and *Gpr12* mRNA expression was barely above background in mouse ovaries when compared to *Gpr12*-null ovaries (data not shown). Signal was also evident on cells in the somatic compartment; however, it is unclear whether this signal is above background level. In situ hybridization performed with the sense probe showed a uniform pattern of distribution of the silver grains throughout the tissue (data not shown).

Expression of Gpr3 and Gpr12 mRNA is dependent on cAMP signaling in the oocyte

Oocytes from the *Pde3a*-KO mice are arrested in meiotic prophase because of persistent high levels of cAMP (Masciarelli et al., 2004). In these oocytes, the levels of cAMP are chronically elevated because there is little or no cAMP-PDE activity. However, the increase in cAMP in the *Pde3a*-KO is modest (less than twofold) in comparison with the increase in cAMP observed when PDE3 is acutely inhibited with cilostamide (data not shown). This suggested that a compensatory decrease in adenylyl cyclase activity and/or signal transduction components is present in these oocytes; such negative feedback regulation involves decreased expression (downregulation) of GPCRs that signal through adenylyl cyclase activation. An increase in cAMP signaling is compensated by downregulation of receptor expression, whereas a decrease in cAMP signaling is followed by an increase in receptor density (Luttrell and Lefkowitz, 2002). We therefore used these genetically altered oocytes to test whether a chronic increase in cAMP has an impact on *Gpr3* and *Gpr12* mRNA expression. To test this possibility, 1000–2000

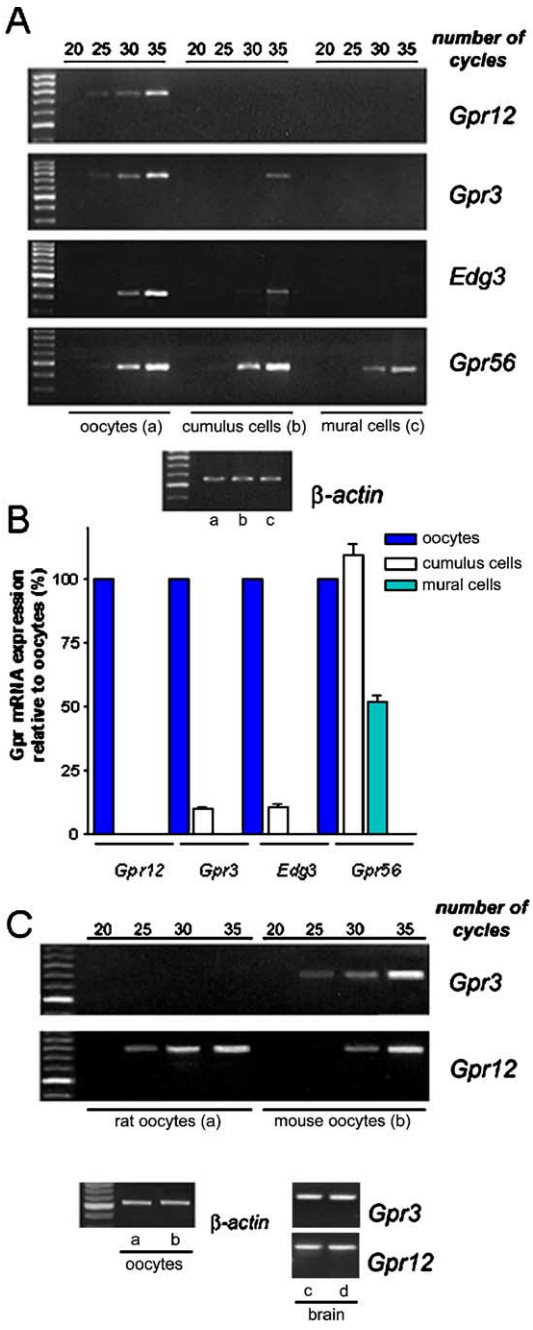

Fig. 2. Expression of GPCR mRNA in oocytes and surrounding somatic cells. (A) RT-PCR of RNA from mouse oocytes, cumulus cells, and mural granulosa cells was performed by using primers specific for *Gpr12*, *Gpr3*, *Edg3*, and *Gpr56*. Control PCRs for β-actin were carried out to ensure that an identical amount of cDNA was used for each cell type and gene. The data shown are representative of RT-PCRs from four different preparations of RNA and cDNA. (B) The intensity of the ethidium-bromide-stained bands was analyzed by NIH Image software. Data are expressed as the ratios of the intensity of each band to the *β-actin* band. The ratio of intensity of the oocyte was set as 100%. (C) *Gpr3* and *Gpr12* expression in rat and mouse oocytes was compared by RT-PCR with gene-specific primers on identical amounts of cDNA. In parallel, PCRs were performed with β-actin primers to monitor for the amount of cDNA from rat (a) and mouse (b) oocytes used. RT-PCR was also performed using rat (c) and mouse (d) brain as positive controls for the primers.

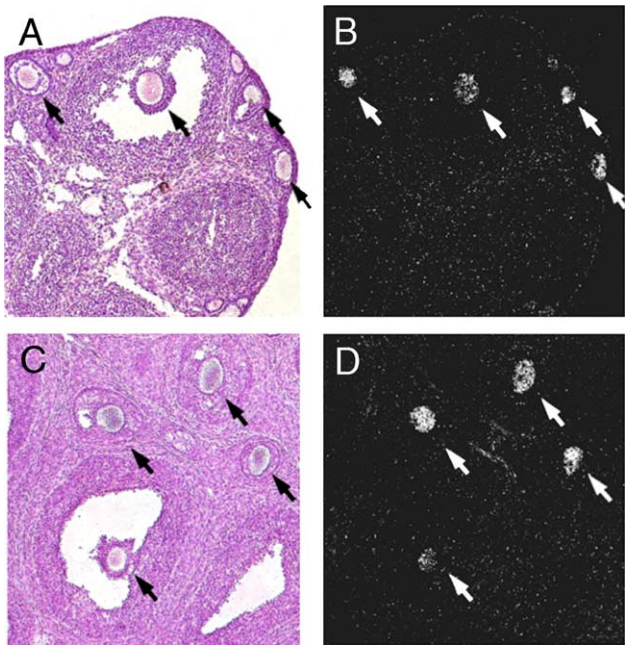

Fig. 3. Pattern of *Gpr3* and *Gpr12* expression in rodent ovaries using in situ hybridization. Ovaries were excised from PMSG-primed immature mice and rats and fixed and sectioned as detailed in the Materials and methods. Mouse (A, B) and rat (C, D) sections were hybridized with antisense *Gpr3* (A, B) and *Gpr12* (C, D) RNAs, respectively, and processed in an identical manner. Sections hybridized with the corresponding sense RNA showed a uniform distribution of the grains throughout the section (data not shown). Arrows indicate oocytes in follicles at different stages of development. Representative sections of the three experiments performed for each gene and species are shown.

denuded GV oocytes from 25-day-old PMSG-primed *Pde3a*-deficient and wild-type littermates were isolated, RNA extracted by the TRIzol method, and the expression of *Gpr3*, *Gpr12*, and *Gpr56* were investigated using semiquantitative measurements (Fig. 4). Whereas *Gpr56* levels were not significantly changed, the expression of both *Gpr3* and *Gpr12* was decreased by more than 50% in the *Pde3a*-deficient oocytes (Fig. 4).

GPR3 and GPR12 display constitutive activity toward Gs when overexpressed in somatic cells and in frog and mouse oocytes

A distinctive feature of the GPR3/GPR12 family is that they show constitutive activity toward Gs when expressed in several heterologous systems (Eggerickx et al., 1995; Uhlenbrock et al., 2002). This property of the two receptors was confirmed by expressing GPR12 and GPR3 in COS7 cells (Fig. 5). Expression of either receptor produced a large increase in cAMP production, whereas the expression of two other receptors linked to Gs and cAMP showed no activity in the absence of ligand (Fig. 5A). The increase in cAMP following GPR3 or GPR12 expression was present whether the transfection was performed with constructs with the native *Gpr* sequence or a fusion with a synthetic signal peptide sequence, suggesting that the constitutive activity is observed at a wide range of receptor expression levels. Upon treatment of the transfected cells with S1P, and to a lesser degree SPC, a small but significant increase in cAMP accumulation was observed (Fig. 5B).

To determine whether this property of GPR3 and GPR12 is sufficient to maintain the meiotic blockade, mouse oocytes were maintained in meiotic arrest with the PDE3 selective inhibitor cilostamide (10 μM) and injected with *Gpr3* and *Gpr12* mRNAs. After 4 h, oocytes were washed free of the inhibitor and spontaneous maturation was observed for the following 24 h (Fig. 6A). Noninjected oocytes or oocytes injected with *Gfp* mRNA underwent spontaneous maturation, whereas, in oocytes injected with either *Gpr3* or *Gpr12*, spontaneous maturation was prevented, with *Gpr12* being less effective than *Gpr3* (Fig. 6A).

In a parallel experiment, the two receptors were expressed in frog oocytes. Unlike mammalian oocytes, frog oocytes do not undergo spontaneous meiotic maturation when isolated from their follicle environment, and progesterone stimulation is required to induce reentry into the cell cycle. Expression of increasing concentrations of either *Gpr3* or *Gpr12* mRNA produced a concentration-dependent block in oocyte maturation (Fig. 6B). Conversely, expression of FSHR had no effect on oocyte maturation induced by progesterone.

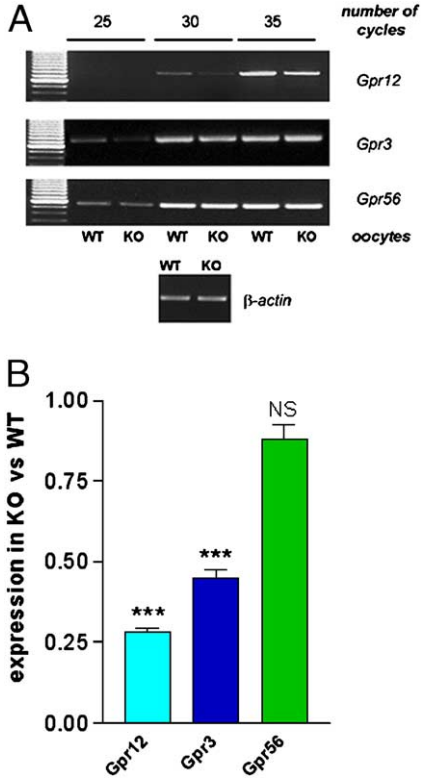

Fig. 4. Downregulation of *Gpr3* and *Gpr12* mRNAs in *Pde3a*-null oocytes. (A) RT-PCR was performed on equal amounts of cDNA from wild-type and *Pde3a*$^{-/-}$ denuded oocytes. The expression of *β-actin* was used as a control. Specific primers for *Gpr12*, *Gpr3*, and *Gpr56* were used for different cycles of amplification. A representative experiment of the three performed is reported. (B) The intensities of the ethidium-bromide-stained bands were analyzed by NIH Image. Data are expressed as the ratio of the intensities of the *Gpcr* to the *β-actin* mRNA. Error bars are the mean ± SEM of three determinations. The decrease in expression of *Gpr3* and *Gpr12* is statistically significant ($P < 0.005$), whereas the expression of *Gpr56* was not statistically different from control.

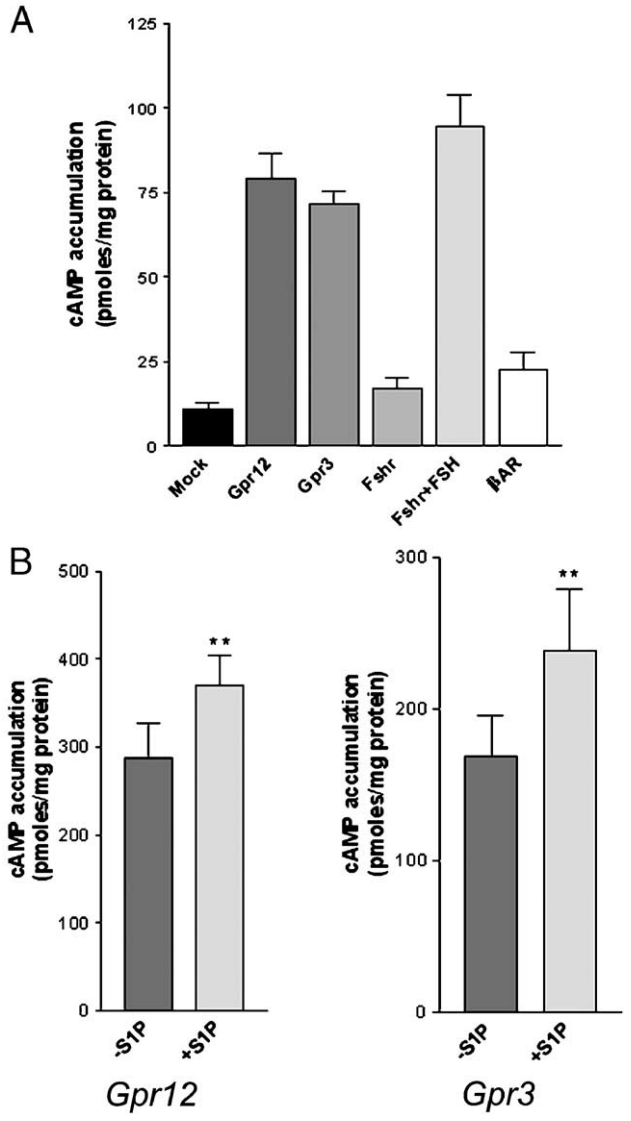

Fig. 5. Increased cAMP levels in cells expressing GPR3 and GPR12. (A) COS7 cells were transfected with either *Gpr3*, *Gpr12*, *Fshr*, or *Adrb2* expression vectors. Empty vector was used as a control. Forty-eight hours after transfection, cells were rinsed and serum-starved for 24 h. At the end of the incubation, cells were harvested in 0.1% TCA in ethanol, and cAMP was measured as detailed in Materials and methods. Each point is the mean ± SEM of three plates. A representative experiment of the three performed is reported. (B) Transfected cells were incubated for an additional 30 min with 0.5 mM IBMX in the absence or presence of 10 μM S1P and were harvested as above for cAMP measurement. Error bars are the mean ± SEM of five separate experiments. Using paired *t* tests, $P < 0.01$ for the effect of S1P on Gpr12 and on Gpr3.

Downregulation of GPR3 and GPR12 by morpholino oligonucleotides affects meiotic maturation

To further define whether GPR3 and GPR12 in mouse and rat oocytes have overlapping functions in meiosis, downregulation of the two receptors was induced using morpholino oligonucleotides (MO) (Fig. 7). Oocytes maintained in meiotic arrest by incubation in hypoxanthine (4.5 mM) were injected with antisense morpholino oligonucleotides corresponding to *Gpr3* and *Gpr12*, and the meiotic progression was followed up to 48 h (Fig. 7). In the mouse, *Gpr3MO* injection caused oocyte maturation (Fig. 7A), whereas *Gpr12*MO was ineffective. Conversely, *Gpr12*MO but not *Gpr3MO* produced meiotic resumption in rat oocytes (Fig. 7B). Most of the oocytes injected with scrambled oligonucleotides and the noninjected oocytes remained arrested in GV stage for the entire culture period, similar to that previously observed with hypoxanthine (Eppig et al., 1985). The effect of GPR3 knockdown in the mouse is consistent with the mouse *Gpr3* knockout data (Mehlmann et al., 2004). This species-specific difference in GPR3 and GPR12 downregulation underscores the importance of GPR12 in the rat and strongly suggests that the two receptors have complementary functions.

A ligand for GPR3/GPR12 delays spontaneous meiotic maturation

To test whether a GPR3/GPR12 ligand impacts meiotic maturation, ovaries were excised from immature PMSG-treated mice. Oocytes were isolated in the absence or presence of 1–10 μM SPC or S1P, or 10 μM sphingomyelin (SM) as a negative control, and the time course of meiotic maturation was followed using the time of follicle puncture as 0 time (Figs. 8A, B). Oocytes collected in 10 μM SPC or S1P showed a consistent and significant delay in oocyte maturation, whereas oocytes collected in SM, a phospholipid present in the lipid rafts of the plasma membrane but not thought to be a ligand for GPCRs, matured at the same rate as control oocytes. In the five different experiments performed after isolation in the presence of 10 μM SPC, maturation at 75 min was only 13% compared with 59% in the control oocytes ($P < 0.001$) (Fig. 8B). Experiments done with S1P produced similar results with significant inhibition ($P < 0.05$) although more variable, possibly because of the physical properties of the compound. These experiments suggest that the activity of GPR3 and GPR12 expressed in the oocyte is potentiated by a ligand.

Impact of genetic Gpr12 ablation on oocyte maturation in vivo

The data described above are consistent with the hypothesis that expression of GPR3 and GPR12 in oocytes is involved in maintaining cAMP levels sufficient for meiotic arrest. This possibility was further tested by investigating whether a leaky meiotic arrest follows *Gpr12* ablation in mice. However, analysis of the meiotic stage of oocytes in antral follicles from two PMSG-treated immature and two adult *Gpr12*-null mice showed no signs of precocious maturation. Oocytes present in antral follicles had a well-formed GV. This indicated that the constitutive activity of GPR12 is not required to maintain meiotic arrest by itself and/or that compensation by GPR3 occurs in the *Gpr12*-KO mice. Ablation of *Gpr3* produces a phenotype of precocious meiotic resumption (Mehlmann et al., 2004).

Discussion

With the present study, we provide evidence for the expression of an array of GPCRs in rodent oocytes, opening

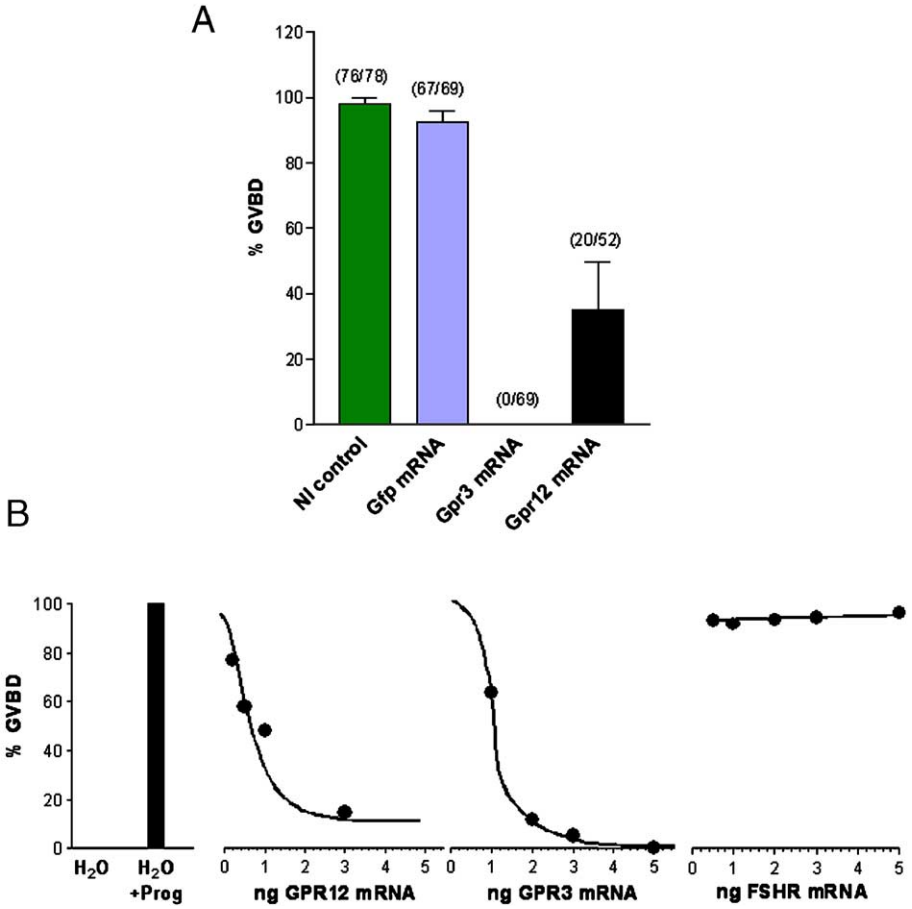

Fig. 6. Expression of GPR3 and GPR12 in mouse and frog oocytes blocks spontaneous or progesterone-stimulated meiotic resumption. (A) Denuded mouse oocytes maintained in GV with 10 μM cilostamide were injected with *Gfp*, *Gpr3*, or *Gpr12* cRNA or not injected (NI control). After 4 h, oocytes were washed and placed in medium without cilostamide under oil and scored for maturation up to 24 h. Percentage maturation is shown for oocytes injected in three different experiments (mean ± SEM). (B) Stage VI *Xenopus* oocytes were injected with increasing concentrations of cRNA coding for *Gpr3*, *Gpr12*, or *Fshr*. Twelve hours after the injection, oocytes were treated with 500 nM progesterone, and oocyte maturation scored after 6 h. A representative experiment of the three performed is reported.

the possibility that the female gamete is able to adjust its functions to numerous extracellular cues present in the follicular environment. In view of the properties of GPR3 and GPR12 and their pattern of expression in the follicle, we propose that these receptors are involved in the control of cAMP accumulation in the oocyte and are likely involved in the maintenance of meiotic arrest. That GPR3 and GPR12 are implicated in cAMP signaling in oocytes is consistent with the observation that their mRNA expression is reduced when cAMP is chronically increased in oocytes. Because the constitutive activity of these receptors is sufficient to prevent maturation in mouse and *Xenopus* oocytes, we surmised that their activity is also sufficient for maintaining the meiotic arrest in the follicle. In view of the absence of an oocyte meiotic maturation phenotype in the *Gpr12*-null mice, we inferred that GPR3 is the predominant receptor signaling meiotic arrest in mice. Conversely, it is likely that GPR12 plays a predominant role in meiotic arrest in rat oocytes. Indeed, in vitro data of downregulation of GPR3 and GPR12 in mouse and rat oocytes using morpholino-modified oligonucleotides are consistent with this hypothesis. Thus, our findings complement and extend a recent report that meiotic arrest is defective in mice deficient in *Gpr3* (Mehlmann et al., 2004).

More than 30 GPCR mRNAs were detected in mouse and rat oocytes by the three strategies used. The expression of *Gpr3* and *Gpr12*, as well as *Gpr56*, was detected by microarray analysis, RT-PCR with degenerate and gene-specific primers, as well as by EST database scanning. *Edg3* expression was detected by microarray analysis and confirmed by RT-PCR. The expression of the other GPRC genes is inferred from the microarray data and, in some instances, by EST retrieval from oocyte or egg libraries. Although the expression of these additional GPCRs needs to be further confirmed, it is likely that several seven transmembrane receptors coupled to different G proteins are functional in oocytes. In many instances, the ligands for these receptors are not known, as for GPR1, GPR19, and GPR56, as well as for the olfactory receptors. GPR56 is a secretin-like receptor implicated in brain development as inferred by linkage with bilateral fronto-parietal polymicrogyria (Piao et al., 2004), and *Gpr56* mRNA has been previously detected in oocytes (Stanton and Green, 2001). Here, we have confirmed this expression by RT-PCR, as well as by microarray and EST database scanning. In addition to its presence in the oocyte, *Gpr56* mRNA is also detected in mural granulosa and cumulus cells by RT-PCR with specific primers. This receptor is

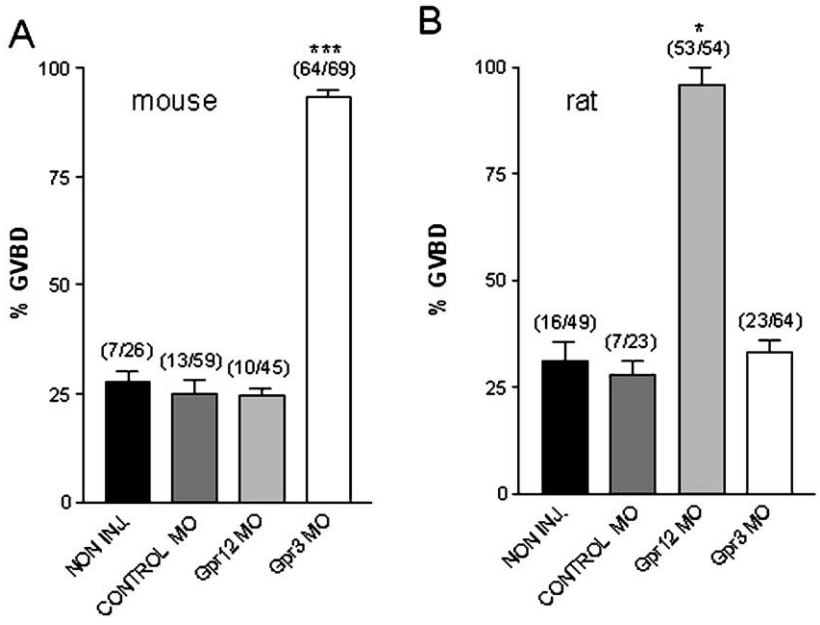

Fig. 7. Effect of downregulation of GPR3 and GPR12 on mouse and rat oocyte meiotic arrest. Oocytes were collected in medium supplemented with 4.5 mM hypoxanthine to prevent spontaneous maturation. Groups of oocytes were injected with either a scrambled morpholino oligonucleotide (control oligo) or with morpholino oligonucleotides corresponding to *Gpr3* (*Gpr3MO*) or *Gpr12* (*Gpr12MO*). A group of noninjected oocytes was used as a further control for the injection. Oocytes were maintained in medium with hypoxanthine for up to 48 h and the state of meiotic progression evaluated by Hoffman interference microscopy. Data are reported as percentage of oocytes that resumed meiosis (GVBD); the numbers above each bar represent the total number of oocytes injected in at least three separate experiments done on different days with different batches of oocytes (***$P < 0.001$).

coupled to Gq (Little et al., 2004) and therefore does not directly signal through cAMP; thus, its function in the oocyte remains to be determined. Conversely, adenosine effects on oocyte maturation have been previously reported (Salustri et al., 1988; Tornell et al., 1990; Eppig et al., 1985). It is then likely that these effects are exerted through the A2A and/or A2B receptors, whose expression we have detected by two approaches. However, the exact physiological significance of this expression is unclear as the *Adora2a*-KO mice do not display a fertility phenotype (Ledent et al., 1997).

The expression of *Gpr3* and *Gpr12* mRNAs was detected by RT-PCR in denuded mouse oocytes. That the detection of these mRNAs is the consequence of contamination from somatic cells is unlikely, in view of their low level of expression in the somatic compartment. Moreover, in situ hybridization showed a predominant signal on the oocytes with *Gpr3* probes, whereas the signal on cumulus or mural granulosa cells was low and difficult to distinguish from background. Rat oocytes expressed *Gpr12* mRNA, whereas *Gpr3* mRNA could not be detected either by RT-PCR or in situ hybridization. Because of the different properties of the sets of primers used, it is not possible to accurately assess the relative steady state levels of the *Gpr3* and *Gpr12* mRNAs by RT-PCR in oocytes nor can microarray data provide absolute quantitative measurements. Nevertheless, on the basis of all our findings, it is tempting to propose that *Gpr3* is expressed at higher levels than *Gpr12* in mouse oocytes with a ratio of 4:1, whereas *Gpr12* is the predominant receptor expressed in rat oocytes. These different levels of expression are consistent with the morpholino effects and may explain the absence of a meiotic phenotype in the *Gpr12*-null mice.

An involvement of GPR3 and GPR12 in the control of cAMP levels in oocytes is consistent with the observation that the expression of these receptors is decreased in the *Pde3a*-null mice. These oocytes are arrested in meiotic prophase because of a persistent increase in cAMP that follows PDE ablation (Masciarelli et al., 2004). We observed that the increase in cAMP in the *Pde3a*-KO is modest (less than twofold) in comparison with the increase in cAMP observed when PDE3 is acutely inhibited with cilostamide. This finding is consistent with the observation that the mRNA for *Gpr3* and *Gpr12* is decreased in these oocytes. We propose that the chronic increase in cAMP that follows PDE3A ablation triggers a compensatory downregulation of the receptors coupled to Gs and adenylyl cyclase in the oocyte. This is a well-established regulatory loop in somatic cells essential for the control of receptor density and endocrine homeostasis (Luttrell and Lefkowitz, 2002).

Overexpression of GPR3 and GPR12 in COS7 cells causes a large increase in cAMP accumulation, a finding consistent with previous reports (Eggerickx et al., 1995; Uhlenbrock et al., 2002). Similarly, overexpression of either receptor in frog and mouse oocytes prevents progesterone-induced maturation and spontaneous maturation, respectively. This finding strongly suggests that these receptors have sufficient intrinsic activity to maintain meiotic arrest and have largely overlapping properties. However, *Gpr12* gene inactivation in the mouse fails to produce a phenotype of precocious oocyte maturation, whereas *Gpr3* inactivation causes a clear phenotype of leaky meiotic arrest (Mehlmann et al., 2004). Although the level of expression of the corresponding proteins could not be assessed in oocytes, differences in concentrations of the two receptors in

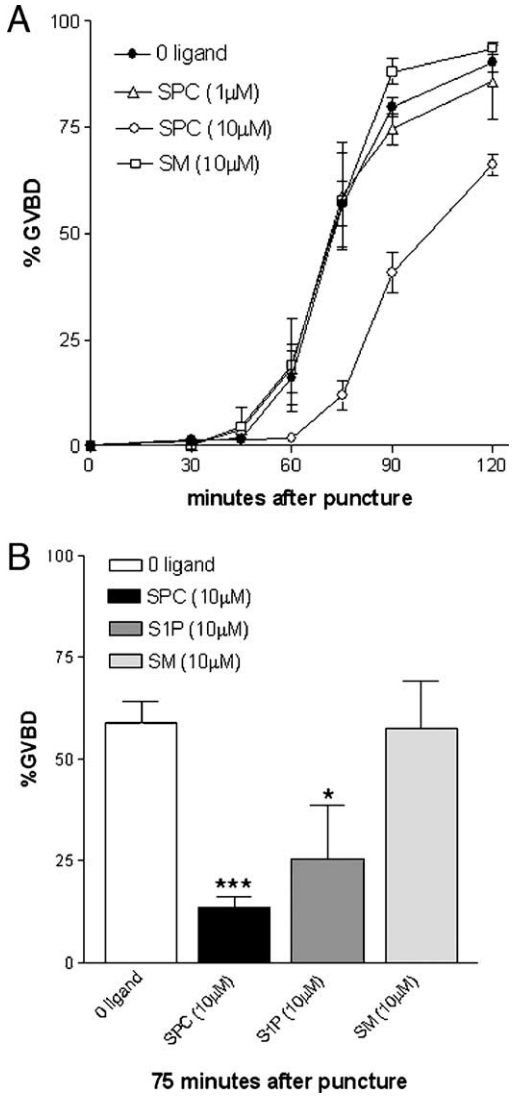

Fig. 8. Effects of SPC, S1P and SM on the time course of spontaneous maturation in mouse oocytes. (A) Denuded oocytes were collected in L-15 medium with 0.7% PVP with or without 1 or 10 μM SPC or 10 μM S1P or SM and then placed in 25 μl drops under mineral oil. Oocytes were scored for meiotic maturation as described in Materials and methods with time 0 set as the time of puncture of follicles. (B) Summary of the stage of maturation of oocytes treated with 10 μM SPC, S1P, or SM 75 min after isolation. The differences between the control and SPC and S1P groups at 75 min are statistically significant ($P < 0.001$ and $P < 0.05$, respectively). The data represent the mean ± SEM of 4 to 7 experiments.

the mouse may explain the divergent effect on oocyte maturation. In the same vein, GPR12 is most likely the GPCR involved in maintaining meiotic arrest in rat oocytes. Whereas *Gpr12* mRNA was detected in both mouse and rat oocytes by RT-PCR, *Gpr3* mRNA could not be detected in rat oocytes. Assuming that the two receptors have similar functions, we predict that in vivo downregulation in rat oocytes should produce an effect similar to that produced by the *Gpr3* homologous recombination on mouse oocytes. Indeed, our data on downregulation of GPR12 in rat oocytes in vitro are seemingly consistent with this hypothesis. Thus, GPR12 plays a role in the control of meiotic arrest in the rat and possibly in the mouse. Interestingly, the number of competent oocytes undergoing premature maturation in the *Gpr3*-null adult mice is 60–70% (Mehlmann et al., 2004) opening the possibility that another receptor, likely GPR12, compensates for the loss of GPR3. Similarly, the absence of a meiotic phenotype in the *Gpr12*-KO mouse may be entirely due to compensation by the GPR3 receptor expression. Finally, *Edg3* ablation produces only a minor decrease in litter size, even though the mRNA for this receptor is detected in oocytes (Ishii et al., 2001). It remains to be determined whether subtle effects on meiotic arrest are present in females deficient in this gene. It is unclear whether EDG3 receptors affect cAMP signaling. EDG3 produces only a small increase in cAMP when expressed in a heterologous system, and pertussis toxin inclusion is required to detect this effect, suggesting that also this receptor may signal through Gs (Kon et al., 1999). Thus, a role for EDG3 in the control of cAMP levels in oocytes cannot be excluded at this point.

Together with the EDG (also known as $S1P_{1-5}$) subfamily, GPR3, GPR6, and GPR12 are a subfamily of GPCR receptors that recognize sphingosine-1-phosphate or sphingosylphosphorylcholine as ligands. These are lipid mediators known to have a wide array of pleiotropic effects in the most diverse regions of the body, including effects on cell migration, angiogenesis, immune cell function, and cardiac and central nervous system development (Kostenis, 2004). Sphingolipids are ubiquitous constituents of the eukaryotic membranes. They include the acylated sphingoid base ceramide and sphingosine-1-phosphate, which is generated via phosphorylation of sphingosine by a sphingosine 1 kinase (Hannun et al., 2001). In frog oocytes, ceramide causes meiotic resumption (Strum et al., 1995) and progesterone affects lipid synthesis (Morrill and Kostellow, 1999), suggesting that metabolites of these lipids may function during meiotic maturation in frogs. S1P has been shown to prevent apoptosis of oocytes exposed to chemotherapeutic damage (Morita et al., 2000). In view of their ubiquitous presence, it is most likely that S1P and related ligands are present in the follicular environment and accessible to the oocyte. Thus, it is possible that GPR3/12 occupancy by these ligands contributes to maintain meiotic arrest in oocytes in the follicle. The decrease in ligand concentration when the oocyte is removed from the follicular environment may decrease GPR3/GPR12 activity, allowing spontaneous maturation. However, the two putative ligands we used had modest effects on oocyte maturation when added at concentrations up to 10 μM. This incomplete effect is consistent with the finding that these ligands produce only a small increase in cAMP accumulation when GPR3 or GPR12 are expressed in a heterologous system. A caveat in the interpretation of these findings is that the ligands may be rapidly metabolized under the culture conditions used. It also cannot be ruled out that they may be sequestered into lipophilic compartments or bound to a carrier protein. The experiments were performed in the absence of serum, thus decreasing the possibility of carrier protein interference. The zona pellucida, however, could represent a barrier for the diffusion of these ligands. Therefore, whether lipid binding to GPR3 or GPR12 contributes to the prevention of meiotic maturation remains an unresolved issue.

Alternatively, these receptors may function exclusively by virtue of their constitutive activity in the absence of a ligand, and the interaction with Gs may be sufficient to increase cAMP to levels that preclude maturation of the competent oocyte within the follicle. The reason why this activity is not sufficient to prevent spontaneous maturation is unclear at present. We have shown that during spontaneous maturation an increase in PDE3 activity is detected in the oocytes (Richard et al., 2001). This increased activity may be sufficient to overcome the constitutive activity of the two receptors. Furthermore, it has been previously proposed that oocytes do not produce sufficient cAMP and gap junctions are essential for maintaining meiotic arrest by allowing diffusion of cAMP from the somatic compartment (Dekel et al., 1981). According to this model, meiotic maturation occurs because of closure of the gap junction following the LH surge (Sela-Abramovich et al., 2005). It is possible that cAMP diffusion plays a role in both meiotic arrest and meiotic maturation and that this mechanism is complementary to intra-oocyte production of cAMP due to GPR3/12 expression. However, the following findings are in conflict with a primary role of gap junctions in maintaining cAMP in oocytes. *Pde3a*-deficient oocytes are maintained in meiotic arrest even 24 h after release from the follicle, which would not be possible if oocytes did not have the capacity to produce cAMP. Moreover, the effects of *Adcy3* genetic ablation (Horner et al., 2003), as well as inactivation of Gs by antibody injection into oocytes within the follicle (Mehlmann et al., 2002), strongly support the view that the oocyte itself produces sufficient cAMP to maintain meiotic arrest. Further studies are required to assess whether multiple mechanisms intrinsic and extrinsic to the oocyte cooperate in the control of the pool of cAMP that signals meiotic arrest.

We have recently reported that the LH surge activates the expression of EGF-like growth factors and that these factors recapitulate many of the LH effects, including oocyte maturation (Park et al., 2004). These factors do not act directly on oocytes since denuded oocytes maintained in meiotic arrest with hypoxanthine do not mature in the presence of these growth factors, whereas maturation can be induced in cumulus–oocyte complexes. Therefore, it is possible that these EGF-like growth factors modify the cumulus cell function and that these in turn affect oocyte cAMP by modulating the activity of GPR3 and/or GPR12. Indeed, crosstalk between EGF and lipid signaling is well established (Daub et al., 1996), and EGF action has been associated with major changes in lipid metabolism and signaling (Le Stunff et al., 2004). Therefore, a link between the EGF network and GRP3/GPR12 signaling may be possible.

In summary, we have demonstrated that a number of seven transmembrane domain receptors are expressed in mouse and rat oocytes. Of these receptors, GPR3 and GPR12 appear to stimulate Gs activity in a ligand-independent fashion, at least in a reconstitution system. Although we have shown that this property is sufficient to prevent maturation in frog and mouse oocytes and their activity is required to maintain the meiotic arrest in vitro, it remains to be determined how the endogenous receptors function. Their coupling to different G proteins in the oocyte and the effect of ligands on this coupling remain to be determined. In view of the differences in expression of these receptors in mouse and rat, we propose that both GPR3 and GPR12 play a critical role in the control of cAMP levels in the oocyte and in meiotic arrest.

Note added in proof

During the revision of the manuscript, an additional study was published documenting that *Gpr3* ablation produces incomplete meiotic maturation and premature ovarian aging in the mouse. (Ledent C, Demeestere I, Blum D, Petermans J, Hamalainen T, Smits G, Vassart G. Premature ovarian aging in mice deficient for *Gpr3*. Proc Natl Acad Sci USA. 2005 102:8922–8926.)

Acknowledgments

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